

NSF director's last testament

Erich Bloch, for six years director of the US National Science Foundation, has delivered his parting words. It seems that the new director can expect ever harder decisions, especially in areas where science and industrial policy collide.

ERIC Bloch proved a highly successful (perhaps the most successful) director of the US National Science Foundation (NSF) on two counts that matter in Washington: he saw out a full six-year term (the first NSF director to do so for 20 years), and he was capable, with a style that was always assertive and sometimes acerbic, at the task of winning yearly real increases in his budget. That the increases were not sufficient to ensure the "doubling of the budget in five years", promised first by President Ronald Reagan and then by President George Bush, is more a sign of how strapped for cash is the federal government than of any lack of friends.

Bloch was for most of his tenure the only outspoken senior science policy figure in Washington and his prescriptions for US science are thus well known. Last week, he had his chance to deliver a final expression of them to the Senate Committee on Commerce, Science and Transportation, before leaving NSF at the end of August. Bloch repeated his key thesis: the emergence of the global economy means that a nation's competitiveness (a word that featured much in his requests for more funds) is now determined not so much by the availability of natural resources, as by its ability to generate and deploy new knowledge.

Bloch's own schemes for increasing US competitiveness were often controversial, but rarely justifiably so. The most debated of the new institutions he supported were the Engineering Research Centers and the Science and Technology Centers. The idea was to increase support for basic research in engineering and technology at these centres, and to involve industry in their running, so that new knowledge could more easily diffuse into industry and enhance competitiveness. Opponents of the scheme said it moved NSF into "applied" research, where it had no business to be, and signalled the abandonment of NSF's traditional support for individual investigators in favour of support for centres.

Criticisms

Neither criticism was well founded; the centres do not carry out applied research for industry and the total fraction of the budget they consume is far too small (less than 2 per cent) for their existence to indicate a radical change in NSF policy.

More justifiable criticism of NSF may be found in its handling of education. Bloch described to the assembled

senators the seamless continuity of NSF's educational efforts. NSF's mission, he said, is to support "maths, science and engineering education at all levels — kindergarten through high school; undergraduate as well as graduate and postdoctoral studies". But NSF has not always been in the business of supporting science education from cradle to grave. The Reagan administration wiped out the early-education function of NSF, restoring it a few years later when concern about the poor international standing of US high-school students aroused a public outcry. Since then, the extent of NSF's involvement in education has provoked endless bickering, with Congress trying to designate more funds for early education than Bloch deemed appropriate, and with Bassam Shakhshiri, an extremely vigorous head of NSF's Education Directorate, apparently calling for an even bigger investment.

Funding

This difference of opinion appears to have been behind the much-criticized dismissal of Shakhshiri two months ago. Argument behind the scenes has not ended and is probably inevitable; the business of giving out research grants and that of kindergarten education have, after all, little to do with one another — pre-school science education and postgraduate education are not two ends of a continuum. For that reason, NSF would be a lot better off if much of its education function were taken elsewhere and dispute over the allocation of funds between scientific research and early science education were fought out in public, not within an agency which must, inevitably, pretend that such quarrels do not exist.

What of NSF's future? Bloch assured the assembled senators that, "A wealth of opportunities await [*sic*] the next NSF director".

The truth is that times are not only tougher than they have been, but they are more complicated. Some of NSF's difficulties are due to simple bad luck: its budgetary account happens to sit in with those of the Veteran's Administration, Housing and Urban Development and the National Aeronautics and Space Administration, so that any money granted to one is money lost to the others. The total size of the pot from which NSF must fight for its \$2 billion annual budget is about \$65 billion millions, much of it tied up in non-discretionary entitlements. In comparison, the National Institutes of Health,



upon which Congress so regularly showers unasked for gifts, obtains its \$7,000 million from a health bill containing some \$160,000 million, a third of it available in discretionary funds.

Administrative arrangements are only one part of the problem. The underlying difficulty is that no sum of money will long satisfy the demands of the scientific research enterprise. The rate of growth of scientific knowledge is so great that no conceivable expansion of the economy can keep pace and allow support for a high percentage of even the best proposals in every field. And as science has grown, so its importance has changed; as Bloch put it, "the solution of virtually all the problems with which government is concerned — health, education, environment, energy, urban development, international relationships, space, economic competitiveness and defense and national security — depend on creating new knowledge . . .". Two trends thus emerge for the future: ever harder decisions on priorities will have to be made as demand for funds further outstrips supply, and science will increasingly find itself a part of much broader political issues. Both trends were evident at Bloch's farewell hearing. On the one hand, Bloch was asked to state his research priorities: his answer (given somewhat reluctantly) was similar to that given by Frank Press, president of the National Academy of Sciences, two years ago: the supply of trained people comes first, giant projects like the Superconducting Super Collider, the Human Genome Project and the Space Station came after. On the other hand, Bloch was asked what is essentially a political question — his view of the Justice Department's decision to allow the sale of a small US high-technology company, Semi-Gas, to a Japanese competitor, Nippon Sanso (see page 500 of this issue).

The new director of NSF can expect many more such difficult questions in the area where science and industrial policy collide. Bloch has been a vocal supporter of government involvement in the development of 'generic' (or precompetitive) technology, praising Europe's Esprit and Eureka programmes. At the hearing, he went so far as to suggest that the United States might now need to rethink science and technology policy in the same way that it was rethought (or created) at the end of the Second World War. The implication was that the argument that Vannevar Bush used in 1945 to obtain massive federal support for basic research — that it was of the most fundamental importance in building the wealth of nations — now applied equally well to the "critical technologies essential to entire industries and industrial sectors".

Such thinking is music to Congress's ears, as it suggests that money spent on the right kind of research projects can cure America's economic ills. The success of Japan drives this thinking forward. But those in Congress might like to reflect on some figures; Japan's research support to the information industry over the past decade adds up to an average yearly sum of only \$230 million, a fraction of that which the US government already provides. And there are several highly successful Japanese industrial

sectors (such as pharmaceuticals and machine tools) that have never been the beneficiaries of major government-sponsored research projects. The list of differences between Japan and the United States is very long, alas, and the recipe for success that the next NSF director may wish to present to Congress will be hard to find. □

Alternative monarchy

Now that Prince Charles is voicing his opinion, he should perhaps be taken less seriously.

LIKE Moses from the mountain, Prince Charles has returned from the ordeal of his polo accident with a shining vision of British health care — a vision which he put before the delegates of the conference on Quality Care for Inner City Practices at the Royal Society of Medicine last week. Enlightened by a hospital stay which must surely have been one of the least representative of the state of the health service since his wife's last '*accouchement*', the Prince used his position as patron of the Marylebone Centre Trust to urge the medical community to show more respect to those of their colleagues involved in what he calls "complementary medicine".

This friendly euphemism refers, unsurprisingly enough, to what most of the medical community generously terms "alternative medicine", a contemporary phenomenon which, along with modern architecture and the linguistic desecration of ancient religious texts, forms part of a hard core of Royal bugbears with which various professional institutions are regularly berated.

No-one, however, must overestimate the comfort of the Prince's situation. Despite having shown himself to be not wholly unworthy of the attention and respect that his birth so indiscriminately conferred, the inherent influence of his social position, coupled with its modern charter of dissociation from 'issues', makes the expression of any personal opinion almost inevitably controversial. What a shame then that the most responsible and up-to-date agent of Royal authority in living memory should break his impartial silence on behalf of alternative medicine — medicine as a whole being a field in which he is, by his own admission, an "interested amateur with no training".

Even though he makes light of the "freakish interests and obsessions" that the Press has saddled him with — "pseudo-Buddhist spiritual techniques", "grazing on organic vegetables from my own garden" and so on — one can almost hear the swish of the Prince's phantom beard as he calls for the implementation of such concepts as "health-promoting design": architecture which radically attacks the "spiritual" problems facing doctor and patient, such as the "loss of a sense of community and worship that used to exist". Maybe if the Prince were actually forced to wear a pantomime beard during all such addresses, he would be taken less seriously. Who knows? Whatever the ultimate solution turns out to be, something must be done soon. □

Privatized grief for Italian biotechnology

- Sclavo centre's poor commercial record
- DuPont link unrewarding

London

ONE of Italy's foremost biotechnology research centres is set to close, following its sale into the private sector. The news has saddened Italian scientists working abroad, who say the closure is symptomatic of the Italian government's failure to build on its investment in science — a tendency that has led many of Italy's leading researchers to leave the country.

The Sclavo Research Centre, in Siena, Tuscany, was chosen in the early 1980s for a major investment in biotechnology, with funding from the Italian science ministry and through the state-owned chemicals company Enichem. Although attached to Sclavo SpA (a subsidiary of Enichem producing vaccines, diagnostics and blood products) the centre established a good reputation for basic research, and attracted several leading Italian molecular biologists back from posts abroad. Sclavo now has an international reputation for its work on genetically engineered whooping cough vaccines.

But Sclavo SpA, together with the research centre, was sold last month to Marcucci, an Italian pharmaceuticals and broadcasting group. Marcucci, with little interest in basic research, says the research centre will be closed. Marialuisa Melli, who joined the centre in 1984 from the European Molecular Biology Laboratory in Heidelberg, says that most of the 80 research staff expect to be made redundant later this month. The Sclavo staff have appealed to the Italian president, Francesco Cossiga, to intervene, asking for the centre to be brought back into the public sector, and run as a government laboratory.

Franco Celada, an immunologist now at New York University, describes the Sclavo centre's closure as "heartbreaking". He says that Italian science spending has been hampered by the government's regional policy, with continual attempts to establish research institutes in the impoverished south of the country, most of which have failed. At the same time, successful laboratories have suffered through lack of continued funding.

Celada says the situation is compounded by Italian industry's short-sighted view of research spending. "It seemed that Sclavo was the exception", Celada says, having built up a large and successful research group, but the usual Italian pattern has now prevailed. He notes that the closure coincides with plans

to start up seven new research institutes, including two in Sardinia, with research interests very similar to Sclavo's.

Renato Baserga, at Temple University in Philadelphia, says that most Italian scientists working abroad want to return, but the vagaries of Italian science policy are a disincentive. Apart from the problems of government intervention to satisfy regional policy, a "suffocating" bureaucracy surrounds the supply of reagents and equipment, he says.

Melli fears that if the Italian government does not act quickly, it will not be possible to prevent the centre's demise. Many of Sclavo's leading workers are likely to receive other job offers, and research teams will be broken up.

The research centre's current plight is the result of the poor commercial performance of Sclavo SpA. Amanda Maxwell, from the trade journal *Clinica* describes the company's recent history as "a real minefield". US Pharmaceuticals giant DuPont took a 50 per cent stake in Sclavo SpA in early 1988. But the joint venture with Enichem (which subsequently evolved into Enimont) disintegrated amid acrimonious exchanges, as Sclavo SpA's profits tumbled.

Enimont said DuPont had not satisfied arrangements for technology transfer, while DuPont said Enimont managers had been unwilling to bring joint projects to

HUMAN GENE THERAPY

Anticancer trial's surprise approval

Washington

A PROPOSAL to treat terminally ill cancer patients with human gene therapy was approved with unexpected rapidity last week by the Recombinant DNA Advisory Committee (RAC) of the National Institutes of Health (NIH). A few hurdles still remain — the experiment must be approved by the NIH director and the US Food and Drug Administration — but are expected to be jumped without difficulty. Principal researcher Steven A. Rosenberg of the National Cancer Institute says clinical trials could begin within days of receiving final approval. He believes RAC took the unusual step of approving the experiment on first examination because it will treat patients with skin cancer who have failed to respond to standard cancer therapies.

The proposal follows on from Rosenberg's earlier gene transfer experiments, which began in May 1989. Tumour-infiltrating lymphocytes (TILs) — cells with antitumour activity — were isolated from a patient's tumour, marked with a gene for neomycin resistance and readministered to the patient. The gene was not intended to confer any therapeutic benefit but simply to allow Rosenberg to track the TILs and show that they homed in on the tumour in the patient's body.

Now Rosenberg intends to use TILs as vehicles to deliver to the tumour site molecules that may help destroy the tumour. He will insert the gene coding for tumour necrosis factor (TNF) — a molecule that has been shown to cause cancers to regress in mice — into TILs in the hope that they will produce enough TNF at the tumour site to cause tumour regression.

Expressing surprise at all the attention being paid to the approval of his human gene therapy experiment, Rosenberg says "it is important only if it works".

Diane Gershon

Under-cover advice

London

THE annual advice to the UK Department of Education and Science (DES) from the Advisory Board for the Research Councils (ABRC) on requirements for the DES science budget, will no longer be published. Secretary of State for Education and Science John MacGregor said the change brings the ABRC's advice in line with that from the higher education funding councils.

The ABRC's advice on the science budget has been published since 1982, an isolated instance of public access in the web of secrecy surrounding the annual review of British public expenditure. In future, ABRC is expected to publish a more general document, outlining priorities for British science.

Peter Aldous
■ Michael Fallon did not, as was expected, take over Robert Jackson's responsibilities for science and higher education in the recent ministerial reshuffle. The new science minister is Alan Howarth, previously DES minister for schools.

fruition. DuPont eventually sold its stake back to Enimont in June this year. But this proved to be a temporary measure, ended by the Marcucci deal.

As *Nature* went to press, it emerged that the San Raffaele hospital in Milan has expressed an interest in the Sclavo research centre. San Raffaele is planning an expansion of its research in biotechnology. Melli says that Sclavo researchers will meet San Raffaele representatives this week, but the precise nature of the hospital's proposals are not yet known.

Peter Aldous

Veterans sue to force study

Washington

Two US veterans organizations last week filed suit to force the government to complete a study on the effects that exposure to Agent Orange had on Vietnam war veterans. Although the US Centres for Disease Control (CDC) abandoned the project after saying it could not be done, the two veterans organizations say that any refusal flaunts the explicit directions of Congress.

The two organizations — the American Legion and the Vietnam Veterans of America — say that government scientists were pressured to reject the study in 1987 because of fears that a link between exposure to the chemical defoliant Agent Orange, which contains dioxin, and cancer could leave the government open to lawsuits from private citizens.

The suit is based on a 1979 law mandating a federal study. CDC were assigned to do the work but in 1982, after a four-year effort costing at least \$43 million, they concluded that existing service and Agent Orange spraying records were inadequate to determine how much dioxin each serviceman was exposed to. The White House later cancelled the study.

In April, the US Veterans Administration concluded that although no link between Agent Orange and cancer could be determined, Vietnam veterans stationed in areas that were not sprayed with the defoliant do appear to demonstrate a higher incidence of one particular form of cancer known as non-Hodgkin's lymphoma. It attributed the disease to an unspecified "Vietnam experience" and agreed to pay damages for such cases (*Nature* 343, 478; 5 April 1990).

But the veterans groups believe that a connection between Agent Orange and cancer does exist, something they say a well-conducted study would show. "We're saying the study can be done. It doesn't take a lot of brains to see that if you know where the Agent Orange was sprayed and you know where the troops were stationed, you can put the two together," says American Legion spokesman John Mimmick. The groups want a university or private contractor — but not CDC — to do the study.

Legal experts say that the veterans groups may find it difficult to win their case. Although private suits against the US government are not uncommon, victories are rare. Most cases fall down on an "issue of standing" — a legal provision requires a plaintiff to show that he or she was personally injured by the government's failure to satisfy its legal obligations. In the case of the Agent Orange study, the veterans groups will have to prove not only that the study was improperly halted, but that it would have demon-

strated a cancer linkage if it had been allowed to continue, says Robert Kushen, a lawyer on the staff of Judge Jack Weinstein. Weinstein, a New York district judge who negotiated an \$180 million out-of-court settlement between veterans and seven chemical companies that made Agent Orange in 1984.

Nevertheless, at least one congressman has already made his mind up on the matter. Representative Ted Weiss (Democrat, New York), last week released a statement on a soon-to-be-

WEST BERLIN ACADEMY

Help from the East?

Munich

THE West Berlin Academy of Sciences, a recent invention of the right-wing Christian Democrats, was pushed to the brink of collapse in Berlin last month when the Social Democrat-Green government passed a law dissolving it. In desperation, the academy is looking to its equally beleaguered neighbour, the East German Academy of Sciences, for help.

A conservative Christian Democratic government founded the West Berlin academy in 1987 in the face of strident objections from the Social Democrats (SPD), who were then in the opposition. The SPD criticized the academy as a "debating club" with decidedly conservative leanings which they would eliminate as soon as they were able.

But dissolving the academy has not proved as easy as the SPD had thought. It has taken 18 months for the disputed closing order to pass parliament, where it was reluctantly signed on 17 July by parliament president Jürgen Wöhrbe, a Christian Democrat.

Now, the West Berlin academy is preparing a legal challenge claiming that it may not be dissolved on purely political grounds. The case could set a precedent for the expected struggle over the East German academy, which many in the West would like to abolish. The Social Democratic science senator for West Berlin, Barbara Riedmüller-Seel, has said that a unified Berlin would have just one academy, and neither of the two existing ones would be a candidate.

Due to a quirk of West German law, the legal situation of the West Berlin academy is murky. The main argument of the academy — that the government may close a scientific institution only on scientific, not political, grounds — is based on a clause in the West German constitution (*Grundgesetz*). In any other *Land*, the case would pass out of local courts and move quickly to the highest court in West Germany, the Federal Constitutional

released staff report which finds that "CDC covered up the truth about Agent Orange by conducting a study that had been . . . intentionally designed to fail." It recommends that a new study should be conducted.

CDC spokeswoman Gayle Lloyd says the agency stands by its conclusions. And in an as yet unpublished letter to *Time* magazine criticizing a recent article on the subject, CDC director William Roper points out that the decision not to go ahead with the study had been reviewed and approved by Congress's own Office of Technology Assessment (OTA).

Christopher Anderson

Court at Karlsruhe.

But the high court has no jurisdiction over West Berlin, which despite the presence of numerous West German institutions remains a protectorate of the Allied powers. West Berlin is expected to become part of West Germany later this year when the German states are unified, but that may be too late for the academy.

At the very least, the West Berlin academy hopes the local court issues a stay of execution while the case (and the appeals that are sure to follow) is being heard. If not, the academy has until 31 December to pack up its tent and go.

In order to keep all options open, West Berlin academy president Horst Albach will this week discuss a possible merger with his counterpart from the East German academy, Horst Klinkmann of Rostock. Klinkmann is trying to save at least the learned society section of the East German Academy, originally founded by Leibniz in 1700 as the Prussian Academy of Sciences.

The former Communist government in East Germany made the academy the largest research organization in the country, with dozens of research institutions of its own, in addition to maintaining the learned society.

West Berlin academy spokesman Eberhard Vogt says that any proposed merger would certainly be fraught with problems. "A lot of our members would not want to cooperate" with the East German academy because of its Communist history, he says.

Before the collapse of the Communist regime in East Germany and the move towards unification, the West Berlin academy had hoped to move elsewhere in West Germany to avoid being disbanded (see *Nature* 342, 8; 1989). But the attempts failed and now "we can't leave Berlin", says Vogt, because the city will need help to deal with the economic and environmental challenges it now faces.

Steven Dickman

AIDS IN INDIA

Disaster looms for Bombay

New Delhi

PROMPTED by a warning from the World Health Organisation (WHO) in Geneva, the Indian Council of Medical Research (ICMR) is launching a campaign to fight the spread of AIDS in Bombay. WHO experts have told the council that some 60,000 AIDS patients will require hospitalization in India in the next five years and that Bombay will be worst affected.

According to WHO, the rates of infection in Bombay are "unparalleled". ICMR's own survey in Bombay's red-light district shows that 30 per cent of prostitutes are infected with human immunodeficiency virus (HIV). In WHO's opinion, HIV is being transmitted heterosexually in India.

Based on estimates that there may be 250,000 people infected with HIV in the large cities of India, WHO has warned ICMR that in 1995, "there would be close to 60,000 cumulative AIDS cases even if no new HIV infections occurred after 1989". Although ICMR's own surveillance centres have so far detected only about 3,200 seropositive cases, Dr A. S. Paintal, its director general, says "no one must think for a moment that the predictions of WHO experts are exaggerated". Studies by ICMR show that some 60 per cent of Indian women attending antenatal clinics suffer from inflammations. "The prevalence of inflammation is conducive for transmission of AIDS", says Usha Luthra, deputy director general of ICMR.

According to Paintal, WHO has forecast that "every third housewife of Bombay will be found to be infected with HIV at antenatal examination". Declaring that India is entering a "disaster phase",

Paintal has called for a massive public education campaign and promotion of safer sex practices. Educational institutions, women's organizations and business houses have been asked to assist ICMR in its campaign. Newspapers have been urged to donate space for educational messages to the people of Bombay.

The Ministry of Health, which suffered a 10 per cent cut in its budget this year, is worried about the lack of facilities for people suffering from AIDS. "We had asked ten major hospitals in the country six months ago to create special wards and be ready to treat AIDS patients", says Health Secretary N. Srinivasan. But so far not a single hospital has complied. Last month, the All-India Institute of Medical Sciences, New Delhi's leading medical facility, turned away two people with AIDS because its staff was scared to handle them.

K. S. Jayaraman

■ WHO says that 8-10 million people worldwide are infected with HIV, updating the previous estimate of 6-8 million. The revision follows a detailed analysis of HIV serological data for 1988 and 1989. The new figures reflect the worsening of the epidemic through heterosexual transmission in sub-Saharan Africa, where 5 million people (one in 40 adults) are now thought to be infected, and in Asia. HIV was introduced into Asia only in the mid-1980s, but there are now about 500,000 infected people in the continent.

Michael Merson, director of WHO's Global Programme on AIDS, warns that further revisions are likely, as the HIV prevalence in Latin America and Asia expands rapidly, and the African epidemic continues.

P.A.

SPACE POLICY

Advice from the in-crowd

Washington

FACING renewed criticism of its management and projects, the National Aeronautics and Space Administration (NASA) last week announced the membership of a high-level advisory panel, formed at the behest of vice-president Dan Quayle and the National Space Council, to review the future of the US space programme.

The panel will be headed by Norman Augustine, chairman of Martin Marietta, an aerospace corporation with many NASA contracts. Of the 11 other members, five are from the aerospace industry, two are scientists (James Baker of the Joint Oceanographic Institutes and the Jet Propulsion Laboratory and Louis Lanzerotti of AT&T Bell laboratories) and two are former NASA officials.

Although the panel members are considered knowledgeable and outspoken,

few observers expect that they will suggest major changes in NASA's current system. "This is not a panel that is going to recommend getting rid of the space station, the shuttle or Moon/Mars. These are the elder statesmen of the aerospace industry, not boat rockers", says space analyst John Pike of the Federation of American Scientists. Meanwhile it was reported last week that tests in 1981 had revealed the optical error in the main mirror of the Hubble Space Telescope, but the tests had been discounted because more sophisticated, but now suspect, tests showed no problems. So far there is no evidence that the tests were intentionally suppressed (as had been claimed in the Challenger disaster), but investigators are looking into a 700 per cent cost overrun at contractor Perkin-Elmer during the construction of the mirror.

Christopher Anderson

GENETIC FINGERPRINTING

Forensic tests proved innocent

Washington

As expected, the forensic use of DNA tests (genetic fingerprinting) has won the approval of the Congressional Office of Technology Assessment (OTA). In a report* released last week, DNA tests are described as "reliable and valid when properly performed and analysed by skilled personnel".

Genetic fingerprinting has already been admitted into evidence in at least 185 trials and its validity has never been in serious doubt. All the big disputes over the tests — such as the furore at the Castro murder trial (see *Nature* 339, 501 15 June 1989) — have revolved around the way tests are performed and interpreted.

The report does not attempt to answer the vexing questions of the interpretation of DNA test data. Estimates of the probability that two samples come from the same person depend on knowledge of how similar people normally are — that is, on how genetic markers are distributed in the population. Because people form many different subgroups, and genetic markers are not mixed at random, several court cases are now challenging the assumptions under which a 'match' between two samples was declared.

Alun Anderson

*Genetic Witness: Forensic Uses of DNA Tests, OTA-BA-438 (Washington, DC: US Government Printing Office, July 1990).

AIDS RESEARCH

Mice or men?

San Francisco

A NOVEL project that will use mice implanted with parts of human immune system to test new AIDS drugs has won a \$3.2 million, five-year contract for SyStemic, Inc. of Palo Alto, from the US National Institute of Health. The mice can be infected with HIV and are the only non-human animal to contain functioning human tissues and organs. SyStemics expect that use of the mice will allow faster predictions of how humans may respond to new drugs than other animal models or cell cultures currently provide.

SCID-hu (severe combined immunodeficient-human) mice lack an immune system of their own because of a genetic defect and have had human blood cell precursors, thymus and lymph nodes surgically transplanted into them. They develop functioning human immune systems and when infected with HIV, then treated with AIDS drugs AZT or ddI, their transplanted tissues respond much like those of human patients.

The company began to offer testing of potential AIDS drugs to biotechnology and pharmaceutical firms earlier this year; but this is their first government contract. "We take this as an endorsement of the validity of the model," said C. W. Dickey, a SyStemics director. Elizabeth Schaefer

Japanese bid riles Congress

Washington

EVOKING one of the most sordid political scandals in American history, Senator Albert Gore (Democrat, Tennessee) last week attacked US officials for permitting a Japanese takeover of a US company tied to the government-subsidized Sematech electronics consortium. The twisted tale behind the planned sale — which involved White House intervention and the creation of a complicated systems of checks and balances to insure that the Japanese are not able to obtain Sematech technology — threatens to become an “electronic Teapot Dome”, Gore claimed, with characteristic hyperbole, in a raucous hearing of the Commerce, Science and Transportation Committee.

At the centre of the controversy is Semi-Gas Systems, Inc., a small California-based company that provides very highly purified gas for the production of semiconductor chips. Sematech officials claim that Semi-Gas's technology is two years ahead of any competitor. At least five of the company's key technologies have been developed with the cooperation of Sematech, the US government/industry semiconductor consortium formed to counter Japanese domination of the global semiconductor market.

In April, the Japanese chemicals company Nippon Sanso offered Semi-Gas's parent company, Hercules, Inc., \$23 million for the company. Although Semi-Gas officials strongly opposed the sale, they have so far been unable to raise enough money to buy the company themselves. Last week, an interagency government panel — created to prevent loss of key US technology in just this sort of takeover — approved the sale after recommending an extraordinarily complicated system of protective measures to isolate Sematech technology from Japanese industry.

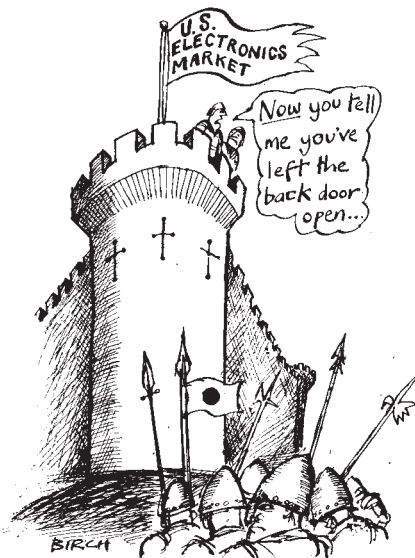
President George Bush has agreed to the panel's recommendation. Although the Justice Department is still reviewing the antitrust implications of the sale — which could give Nippon Sanso as much as 70 per cent of the world-wide high-purity gas market — Sematech officials give the agreement even odds of approval.

After Gore's hearing last week, those odds may have to be recalculated. The senator claimed that the deal raises the possibility that, with as little as \$23 million, a Japanese company may have bought its way into the United States' first-line defence against Japanese domination of the electronics market. Sematech was jointly formed by the US defence department and 14 industrial partners expressly to develop “precompetitive” semiconductor technology that could buoy a US industry faced with

Japanese competitors of apparently unbeatable efficiency.

Although Semi-Gas is not one of Sematech's 14 industrial partners, its technicians work in Sematech's laboratories and much of its technology has been developed with the (taxpayer-subsidized) help of Sematech.

“It's an incredible list of jewels that we are — thanks to President Bush — turning



over to the Japanese”, Gore said. (White House officials dispute this, saying that at least six other companies have technology on a par with that of Semi-Gas, according to Robert Park, Washington director of the American Physical Society). Sematech

NATURAL HISTORY MUSEUM

Reprieves for some senior researchers

London

THE management of the Natural History Museum appears to be backing down slightly from its much-criticized intention to declare some research staff redundant. The NHM's corporate plan provoked unprecedented criticism earlier this year when it declared that one researcher in six would have to be dismissed in order to restructure the museum financially (*Nature* 344, 805; 26 April 1990; 345, 655; 21 June 1990). But three senior researchers faced with immediate dismissal have now been reprieved: archaeozoologist Juliet Clutton-Brock will be allowed to stay until her official retirement, three years hence; palaeobotanist Chris Hill has been offered a three-year fellowship; and the palaeontologist Alan Gentry has been given an extension of a year.

These latest moves risk being seen only as a token gesture to appease the scientific community: at the same time as they were announced NHM director Neil Chalmers reaffirmed his present inclination to sup-

port the commercial exploitation of Museum expertise rather than the development of its “strategic” research. In the latest issue of *Museums Journal* (August, 1990), he says “what most of the world wants from us are good advisory services and not good research”. But just 18 months ago, support for taxonomic research seemed to be at the forefront of his policies (*Nature* 336, 707; 22/29 December 1988).

Chalmers' U-turn is also evident in an internal memorandum circulated in the Museum's Department of Public Services (the arm of the NHM responsible for exhibitions). In the confidential memo, leaked to *Nature*, the Director makes plain that Museum scientists will be required to engage in “corporate” activities. A senior Museum scientist (who declined to be named) said that “corporate activities” account for 5 per cent of Museum researchers' time according to their new job descriptions but nobody seems to know what these activities involve. Henry Gee

tech officials estimate that, if Semi-Gas were to be sold to a Japanese concern, finding a replacement or otherwise protecting Sematech technology would delay current projects by at least six months. Given that Sematech's annual budget is about \$200 million, such a hiatus could cost the consortium as much as \$100 million, they claim.

To prevent Nippon Sanso from gaining access to Sematech's industrial secrets through Semi-Gas, the US Committee on Foreign Investments in the US (CFIUS) is said to have negotiated a complex layering scheme to isolate those Semi-Gas employees who work at Sematech. According to press accounts, the Semi-Gas technicians will report to a special middleman company (controlled by US citizens, including at least one from Sematech), rather than to Nippon Sanso or Semi-Gas directly. Semi-Gas would also be required to protect Sematech documents in a “secure area”.

However, Sematech officials said late last week that they had still not heard from CFIUS, and, in fact, had only learned of a negotiated settlement by reading the *Wall Street Journal*.

Robert White, undersecretary for technology at the Department of Commerce, told Gore that although he had been consulted in the deliberations, he did not know of the confidentiality pact. He pointed out, however, that the act under which the president could countermand such a takeover demands that a rejected bid demonstrate “very clear evidence that it endangers national security”. In the case of Semi-Gas, no such evidence has been shown, he said.

Christopher Anderson

NUCLEAR WASTE

New fears at Hanford

Boston

THE Hanford Nuclear Reservation in Washington state is in trouble again, with the release last week of a report from a US Department of Energy (DOE) advisory panel saying that gases trapped within some of the millions of gallons of highly radioactive wastes stored in underground tanks at the facility could explode.

Government documents unearthed by congressional staff show that DOE contractors knew of the existence of flammable hydrogen gas in at least one tank at Hanford for 13 years, but did not notify higher authorities or attempt to remove the danger until this spring. Warnings of the potential danger of explosion at underground tanks at Hanford and other nuclear production facilities have occasionally surfaced over the past few years.

But no earlier warnings have combined the urgency and authority of the report issued last week by the Energy Department's advisory committee on Nuclear Facility Safety, chaired by John F. Ahearne, former head of the US Nuclear Regulatory Commission.

In a letter sent to Energy Secretary James Watkins last month, before the public release of the report, Ahearne's committee described the tanks as presenting "a serious situation, if not an imminent hazard". Furthermore, the committee wrote, the consequences of an explosion could be grave, owing to "the magnitude of the radioactive inventory available for dispersal".

The problem at Hanford is that a crystallized crust has developed on top of the liquid waste in some of the storage tanks. The crust, which can be as much as two or three feet thick, is believed to have been

caused by chemicals that were added in the late 1950s and 1960s to newly produced liquid wastes at Hanford in order to reduce their volume. Hydrogen gas, which is known to build up in the liquid waste, is now trapped underneath the hardened crust and, according to the report, could be detonated by heat generated inside the tanks or by a spark or shock from outside. The chemical explosion could spread long-lived radioisotopes, including caesium-137 and strontium-90, into the surroundings.

Ironically, Hanford's tank farm was intended to provide only temporary storage for the highly radioactive liquid wastes generated from the processing of plutonium for use in nuclear weapons. Construction began in the 1940s and continued through the 1950s and 1960s as the Hanford facility increased its output of plutonium. But despite the passage of over 40 years, no permanent solution has been found to the disposal problem posed by the liquid wastes. Many of the tanks have leaked.

Dealing with the problems of the Hanford tanks is made more difficult by the lack of knowledge of what is happening inside them. What is certain is that hydrogen trapped in some of the tanks frequently causes so-called 'tank belches' in which the tank's crust moves upwards, sometimes as much as a foot, as it vents the trapped gas. Information released by Senator John Glenn, who chaired congressional hearings on the problem last week, shows that at least three "major steam explosions" have occurred at Hanford's tanks. One explosion, in 1965, lifted a tank six feet off its underground foundation and sent a geyser of radioactive steam 50 feet high into the air.

At the hearing, Glenn said that there is a risk of an explosion comparable to one that occurred in the Ural Mountains of the Soviet Union in 1957, forcing the evacuation of 10,000 people.

DOE officials, however, maintain that the two situations are different, in part because the temperature of Hanford's wastes are believed to be far lower than that which caused the explosion in the Soviet Union.

Privately, DOE may be more concerned about the situation than it says. An inside congressional source said over a month ago that DOE was "frantic" about the situation. A DOE official confirmed that one tank at Hanford has been declared off limits to Hanford workers for over a year because of fears that activity could touch off an explosion. One tank, known as 101-SY, is almost continuously "belching" hydrogen, according to the official, confounding scientific explanations of the phenomenon. **Seth Shulman**

BIOTECHNOLOGY

The long and winding road

San Francisco

CETUS Corporation stumbled on its long road to profitability last week when a US Food and Drug Administration (FDA) regulatory panel refused to grant marketing approval of its most important product, interleukin-2. The surprise announcement sent Cetus stock plummeting, but analysts believe the company is secure enough to weather a delay of several months or more without needing to shed any of its workforce. Approval may be delayed for three months to a year.

Cetus, which is based in Emeryville in California, had high hopes for its first, and only, therapeutic biotechnology product in the near term. It retains however a measure of security from its other endeavors: the three drugs that are in clinical trials, the marketing of generic cancer drugs and the diagnostic application of the company's important polymerase chain reaction technology.

The US FDA's Biological Response Modifiers Advisory Committee asked Cetus to reanalyse some of its data on interleukin-2, which the company planned to offer for the treatment of kidney cancer. Officials of the company hope to meet with the FDA later this month to discuss the recommendation and to determine whether additional testing of the drug will also be required.

Cetus found that the genetically engineered drug caused partial remission in approximately 20 per cent of the patients treated, but the Committee raised concerns over clinical testing methods and side effects. The Biological Response Modifiers Advisory Committee is a new group comprised of nine immunologists who evaluate safety and efficacy data on new drugs and make recommendations to the FDA. Last week, the panel also asked Immunex Corporation of Seattle, Washington, for more study data on the drug GM-CSF (granulocyte macrophage colony stimulating factor) as a treatment for bone-marrow transplant rejection before making its recommendation to the FDA on market approval.

Cetus's President and Chief Executive Officer, Robert A. Fildes, was surprised and disappointed by the FDA decision. "We think we have shown over the past five years that this does bring benefit to a substantial number of people," he said. The drug, which Cetus calls Proleukin, is already approved in nine European countries for the treatment of kidney cancer, and it is in clinical trials in Europe and the United States for treatment of other disorders; Cetus plans to request approval soon in Europe to market Proleukin for the treatment of skin cancer.

Elizabeth Schaefer

FRENCH UNIVERSITIES

Open season

Paris

THE French education minister, Lionel Jospin, is considering setting up a new university, with courses taught through correspondence and the media, on the lines of the successful British Open University which was founded in 1969.

Jospin is currently studying a report by Oliver Duhamel, a lecturer at the University of Paris I, which, while inevitably using the British experiment as a model, nevertheless envisages a less centralized structure. Duhamel proposes a regional organization, linked to state universities and higher education centres, which will also build on existing facilities for learning-at-a-distance. There has been no mention of the amount of time and money needed to establish the venture, but Jospin promised initial experiments in 1992. **Peter Coles**

The return of the natives

Sydney

A DEMAND by an Australian aboriginal group that a collection of ice age human remains, 10,000 to 15,000 years old, should be handed over for reburial has sparked criticisms that the government of the state of Victoria is sanctioning the destruction of priceless relics. The dispute between scientists and members of the Echuca Aboriginal Cooperative, who claim descent from the Pleistocene inhabitants of the Kow Swamp area in northern Victoria, may yet erupt into a legal battle.

The Aboriginal community has told the Museum of Victoria, which holds the remains, that the collection of over 100 bodies must be returned to them according to the provisions of the Aboriginal and Torres Strait Islanders Heritage Protection Act of 1984, under which skeletal remains and sacred sites and artefacts receive federal protection (*Nature* 344, 697; 19 April 1990).

But according to John Mulvaney, secretary of the Australian Academy of the Humanities, the return of such old remains would be unprecedented. "There is no parallel in the world to destroy human remains over 10,000 years old. This is one of the best single examples from the Pleistocene age in existence and the Victorian government is allowing it to be destroyed", said Mulvaney.

Alan Thorne of the department of prehistory at the Australian National University, who excavated the remains between 1969 and 1972 and donated them to the Museum of Victoria in 1985, says that a decision to return the collection has been made by the Victorian Minister for the Arts, Andrew McCutcheon, who is responsible for the museum, the Victorian

Minister for Aboriginal Affairs, Brian Mier, and the Echuca Aboriginal Cooperative. "Nothing has been discussed", complained Thorne, who says that representatives of neither Australian archaeology nor the general aboriginal community have been consulted. "The Victorian government has received more than 200 letters from anthropologists and prehistory experts here and overseas protesting [at] the reburial", he said.

Victoria is divided into regions defined as belonging to aboriginal communities, which can ask for the return of remains taken from their region. But both Thorne and Mulvaney dispute the possibility of a connection between modern aboriginals and the prehistoric Kow Swamp people. "It is nonsense to think that the Echuca aboriginals are direct descendants of a group that lived there 10,000 years ago. These people moved around a lot", Mulvaney said.

Thorne claims that the Kow Swamp site is not part of the Echuca community area, and suggests that a legal challenge to the Victorian government may thus be made, preventing the collection from being handed over. A spokesperson at the Ministry for Aboriginal Affairs agreed that Kow Swamp is "on the border of the Echuca area", and said government lawyers were looking into Thorne's claim.

As a compromise, Mulvaney and Thorne have suggested to the government that the collection be placed in the care of the Echuca aboriginal community to be stored, not buried, so that "white scientists would be denied access but Aboriginal prehistorians and anthropology students could have access to them at a later date".

Tania Ewing

East Germany says no

Munich

EAST Germany may be moving towards a tougher line on the release of even harmless genetically engineered bacteria into the environment, an East German company has discovered to its cost.

Although there are no clear regulations dealing with the release in question, and it had already been pronounced harmless by a local regulatory body, the national Genetics Commission has shut down enzyme production at the company until it conforms to stricter standards.

The 'release', which occurred at the biotechnology company PROWIKO of Schönebeck bei Magdeburg, was actually a continuous trickle of *Bacillus subtilis* bacteria, which were genetically engineered to produce large quantities of the enzyme alpha-amylase. The enzyme is used in food production. The same safety commission had declared the release safe every year since 1986 before deciding to shut it down in March 1990. In West Germany regulation is stricter, but even there the releases might well have been judged harmless.

Waste released from the plant carried the bacteria into the environment, says Kornelia Smalla, laboratory director at the local Magdeburg hygiene inspection office. But the bacteria died almost immediately and their recombinant genes did not persist, she says. Filtering out such bacteria is not required by the guidelines for "good industrial large-scale practice" issued by the Organization of Economic Cooperation and Development (OECD), says Smalla.

An employee of the company, Michael Wölbing, said that stories of the releases had been distorted by both the local and the West German press, which printed "nonsense" about "beer that makes you sick". The company was mystified by the sudden order to close.

New safety demands have been made — such as the requirement to determine the entire sequence and gene products of the plasmid that carries the alpha-amylase gene — that Wölbing finds unnecessarily harsh. The company has stopped making its other products because of a lack of customers.

In contrast to the West, where environmental release has long been controversial, in East Germany there has never been much public concern, says Eva Reich, a physician and a prominent member of the opposition party New Forum in East Berlin. Even now, says Reich, genetic engineering is not yet an issue in most of East Germany. "People still believe in science here", she says.

Steven Dickman

RESEARCH PRIZES

Chain store fortune funds new award

Munich

A LITTLE-known foundation based in the Italian-speaking region of Switzerland will next year offer a prize more lucrative than the Nobel Prize to a researcher in medicine or biology whose achievements "will contribute significantly to improvements in human health". The recipient will receive one million Swiss francs (\$738,000), more than the Nobel Prize's four million Swedish krona (\$688,000). The new award will be granted every second year beginning on 11 October 1991.

The Helmut Horten Foundation was created by the late Helmut Horten (1909–87), a German businessman who founded a successful department store chain in 1936. Despite a challenge from the West German tax authorities, Horten managed to deposit

most of his considerable fortune in Switzerland before he died, childless. The foundation currently has SFr 60 million at its disposal and will receive still more in the near future.

According to the foundation, the award winner must have made "seminal scientific contributions to the basic biomedical sciences, epidemiology, parasitology, or to disease eradication efforts". The winner will be selected by an international committee of experts.

The foundation asks researchers to nominate deserving candidates from anywhere in the world after contacting the Foundation at CH- 6995 Madonna del Piano, Switzerland. The deadline is 15 November 1990. Self-nominations will not be accepted.

Steven Dickman

BHOPAL

Deal is less than final

New Delhi

THE drawn-out dispute between Union Carbide Corporation and the Indian government over liability for the Bhopal poison gas disaster may not be over after all. Although a settlement was reached last year between Union Carbide and the government, then led by Prime Minister Rajiv Gandhi, the new National Front government of Prime Minister Vishwanath Pratap Singh is now seeking to overturn the settlement. Singh's government argues that the settlement was not lawful; Union Carbide contends that it cannot be reviewed just because the government has changed.

More than 3,700 people were killed and tens of thousand injured by the cloud of toxic chemicals that swept out of Union Carbide's subsidiary plant at Bhopal, capital of Madhya Pradesh, in December 1984 in the world's biggest industrial disaster. After protracted litigation that travelled from courts in Bhopal to New York and back to India again, the Supreme Court pronounced a final settlement in February last year.

The settlement required Union Carbide to pay \$470 million in compensation to the gas victims. In turn the Gandhi government — which by a special act of parliament had taken upon itself the right to represent all the gas victims — agreed to drop all criminal cases against Union Carbide and grant immunity against further legal action.

These two clauses in the settlement agreement are now being reviewed again by the Supreme Court. Attorney general Soli Sorabjee informed the court that they were contrary to public policy and amounted to the surrender of sovereign criminal jurisdiction. For its part, Union Carbide describes the government's move as a "political and a legal fraud". The company's counsel, Fali Nariman, argued that Singh's government is bound by the contractual obligations of its predecessor. In any case, according to Union Carbide, the settlement cannot be changed because the Indian government has already appropriated the compensation money paid by Union Carbide.

Because of the renewed litigation, the settlement money paid by Union Carbide in 1989 is still untouched and gathering interest. The Indian government has told the court that the money will not be returned to Union Carbide until the dispute over damages is settled. Union Carbide, however, demands the return of the money with interest if the earlier settlement is struck down by the court. A ruling is unlikely before next month.

K. S. Jayaraman

EUROPEAN COLLABORATION

Faltering steps to superlaser

London

LASER physics is set to be the next field of 'big science' in which European nations will pool resources to compete with the United States and Japan. Senior officials of science funding agencies from the United Kingdom, France, West Germany, Italy and Spain met in London in July to discuss a project to build a 100-kJ ultraviolet laser facility. The instrument will allow European physicists to study plasma behaviour and fusion of hydrogen isotopes, freeing them from the need to buy time at US and Japanese laser facilities.

The meeting, held at the Royal Society in London and hosted by the UK Science and Engineering Research Council (SERC), considered a report describing two alternative technologies. The conservative option, to build a larger version of existing neodymium-glass lasers, would be expensive and relatively energy-inefficient. Newer krypton fluoride excimer lasers would lack these disadvantages and provide for rapid recycling after each firing. But the technology is in an early stage of development, with the largest KrF laser a mere 1-kJ facility at Los Alamos in the United States.

Sigbert Witkowski, director with responsibility for lasers at the Max Planck Institute for Quantum Optics at Garching, near Munich, would prefer to develop a KrF laser, because it is the newer technology, and one in which Europe already has some expertise. But he admits that it is a large step up to a 100-kJ laser. Given the uncertainties, the working group recommended building two intermediate facili-

ties: a 10-kJ KrF laser and a 30-kJ Nd-glass facility. This would allow physicists to test the performance of the two technologies before making a final decision.

Building a 30-kJ Nd-glass laser would give European physicists access to a respectably-high-energy laser as a stop-gap until the larger laser is built. But this may prove unattractive to the five funding agencies on grounds of cost — the laser project will consume up to £25 million a year for ten years. A cost-cutting opportunity has been provided by France, which has offered use of its 20-kJ Phebus Nd-glass laser, sited south of Paris, for comparison with a new KrF facility.

The funding agencies will make a statement on the working group's proposals later this year. Witkowski says a year's design work is needed for the 10-kJ KrF laser, and it would be a further four years before the facility is completed. The 100-kJ laser could be completed on a similar timescale.

Choice of a site for the intermediate KrF laser will wait until funding is agreed, but SERC's Rutherford Appleton Laboratory — where a research team led by Michael Key leads Europe in KrF technology — will be a front runner.

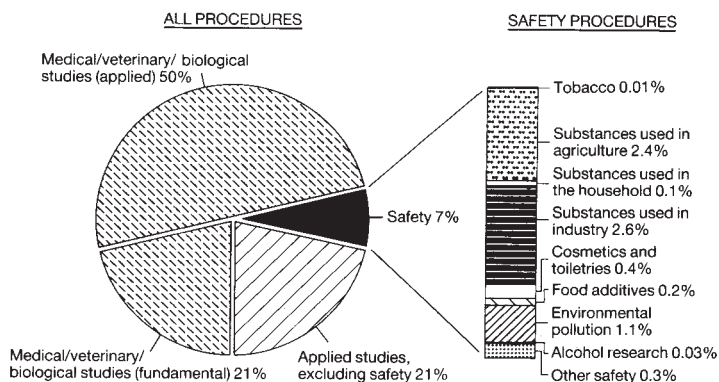
US and Japanese physicists also plan to upgrade their ultraviolet lasers. US physicists are similarly wavering between Nd-glass and KrF technology. One option is to upgrade the Nova Nd-glass lasers at the Lawrence Livermore Laboratories, but the Los Alamos team is also seeking funds for a large KrF laser. Japan is proposing an upgrade of the Gekko Nd-glass laser at Osaka University. **Peter Aldhous**

Trend towards fewer animal tests continues

FOR the thirteenth year in succession, the number of scientific procedures using living animals in the United Kingdom has fallen, to 3.3 million in 1989, about five per cent fewer than in 1988. Contrary to popular opinion, cosmetics testing accounted for only 0.4 per cent of the total, with safety testing of other substances (mostly those used in industry or agriculture) making up another 6.7 per cent, according to figures released by the UK Home Office.

Most licensed procedures (71 per cent) in 1989 were "studies to further medical, veterinary, dental or other biological research", the Home Office says. The applied studies which accounted for 50 per cent of all procedures were dominated by drug development and testing.

Over 60 per cent of procedures in 1989 were carried out in commercial laboratories (mostly pharmaceutical companies) with universities (including medical schools) accounting for another 23 per cent. Smaller numbers of procedures took place in hospitals, polytechnics and government laboratories. Laboratory rodents were used as experimental subjects in 85 per cent of licensed procedures in 1989.



P.A.

Benefits of mission to Mars

SIR—In his criticism of Bruce Murray's Commentary article advocating a manned mission to Mars, N. H. Horowitz (*Nature* **345**, 760; 1990) asserts that no justification exists for such a project that would be commensurate with its cost. Although he may well be correct that the immediate scientific objectives of such a mission could be performed more cheaply with robot spacecraft, there are nevertheless important social, political and scientific arguments that can be advanced in support of an ambitious manned space programme (of which a trip to Mars need form only a part). These include:

(1) The provision of work for the aerospace industry in a disarming world. As the industry employs over 1.3 million people in the United States alone, it will be politically necessary to find an alternative to weapons manufacture. I have argued elsewhere (*New Scientist* No. 1717, p. 67; 1990) that, by providing such an alternative, an expanded space programme may actually be able to assist the process of superpower disarmament.

(2) An expanded space programme would act as a peaceful driving force for the development of new technology and as a stimulus for scientific and technical education. Moreover, by increasing the public awareness of Earth's place in the Solar System, it may help to broaden minds when it comes to tackling important global economic and political problems. Horowitz is scornful of the entertainment aspect of manned spaceflight, but if people are entertained by something, they are likely also to learn from it, and a greater public understanding of our place in the cosmos would be no bad thing.

(3) The provision for a focus for international cooperation, the importance of which for global politics seems to be underestimated by Horowitz. From the point of view of enhancing international solidarity, it is particularly important that cooperation is achieved in areas that are highly visible to the public; space is an obvious candidate.

(4) From the scientific point of view, it can be argued that the long-term interests of astronomy and planetary science will require the construction of large structures in space. For example, the value of a lunar astronomical observatory has recently been reviewed by Burns *et al.* (*Scientific American* **262**(3), 18; 1990). In any case, it is hard to see how human knowledge can fail to advance as a result of the expansion of people into the Solar System.

Thus, rather than opposing a manned space programme on grounds of cost, it seems to me that scientists should acknowledge its potential value, but argue that it should be financed by a transfer of

resources from the military sector of the economy. It is fortunate that the ending of the Cold War has not only made this more realistic politically, but has also left the aerospace companies looking for new projects with which to fill their order books.

IAN CRAWFORD

Department of Physics and Astronomy,
University College London,
Gower Street,
London WC1E 6BT, UK

A funny old game

SIR—Quantitative estimates may help to support your editorial plea for higher scores in Association Football (*Nature* **346**, 92; 1990). But first, how well does the Poisson distribution represent the number of goals scored in matches between approximately evenly matched teams? The table shows the numbers of goals scored per team per full-time (90-minute) game

Number of goals	Observed frequency	Expected frequency
0	32	35.1
1	47	38.1
2	17	20.7
3	2	7.5
4	4	2.0
5	2	0.4
>5	0	0.1
Total	104	103.9

in the 52 matches of the recent World Cup series, together with the numbers expected assuming a Poisson distribution with a mean of 1.09, as actually realized.

A perfect fit is not of course to be expected, as all teams are not approximately evenly matched. Nevertheless, the agreement seems reasonable and if, as is conventional, small numbers are eliminated by pooling the last four rows, the differences are not statistically significant ($\chi^2 = 3.4$, 3 d.f., $P > 0.2$).

Accepting the Poisson model as reasonable, then, the implications of a mean scoring rate of about 1 goal per team per 90-minute game are (1) that the probability of a draw is about 1 in 3 almost irrespective of the relative strengths of the two teams and (2) that if team A is twice as strong as team B in the sense that they have 'expected' scoring rates against each other of 1.33 and 0.67 respectively, then the probability that team A wins a particular match is only slightly greater than 1 in 2. On the other hand, with a mean scoring rate of 4 goals per team, the probability of a draw would fall to about 1 in 9, and the probability that team A (with mean 5.33) beats team B (with mean 2.67) would increase to nearly 80 per cent.

There is little doubt that such an increase in scoring rate would make the

game both fairer (in the sense of producing the right result) and more rewarding to watch and play. The ruling bodies of the sport would do well to ponder these mathematical facts of life, but the implications for the football pools are another matter altogether. . . .

ANDREW D. CAROTHERS

23 Dick Place,
Edinburgh EH9 2JU, UK

Help for Romania

SIR—Your survey of science in Romania (*Nature* **344**, 613–615; 1990) was generally accurate, but some references to Elena Ceauşescu need correction. In fact, she was never called "Professor". This was the only title she could not get in this country and that may be why she hated us, the professors, so much. As a result, she used her unlimited powers to stop all promotions to this academic title in the past 12 years or so. The title she boasted and which was used by the media was "academician doctor engineer", which sounds ridiculous. The addition of her name on research papers in polymer chemistry reporting work done by others is perhaps one of the greatest frauds in science, and all electronic databases should be corrected accordingly. Unfortunately, the printed version of *Chemical Abstracts* and so on cannot be corrected.

In spite of the difficulties of academic life in Romania during the years of dictatorship, there were many scientists who continued to carry out fundamental research (with almost no money), to co-operate with foreign colleagues (often illegally), to attend international meetings and to publish papers in international journals and even books. (I myself managed with great difficulty during this period to have four books published in the West.) Perhaps this could be called "scientific dissidence".

I have three suggestions for ways in which our colleagues in the West could help scientists in Romania and elsewhere in Eastern Europe:

(1) Send reprints of publications to people working in the same field in Eastern Europe without waiting for a request.

(2) If you have sophisticated equipment, provide opportunities for joint research and collaboration.

(3) If somebody in Eastern Europe manages to get a travel grant or a research fellowship, offer him the opportunity to work in your laboratory or research group.

None of these gestures would entail a heavy financial burden, but they would make all the difference to researchers in Eastern Europe.

IONEL HAIDUC

Babes-Bolyai University,
R-3400 Cluj-Napoca,
Romania

Laymen in scientists' clothing?

SIR—We were glad to read Rosner's¹ documented evidence on the pervasive and drastic increase in assertive sentence title (AST) usage in the biological sciences in the past few years. The author proposes that the motivation for such social-psychological strategies of advertisement may be pragmatic, that is, it "lies in the increasing pressure for science to be goal oriented". We suggest that this is true only in those few cases in which Rosner has noticed that the title's bold conclusion is stated in less certain terms elsewhere in the article. We believe that the majority of ASTs reflect a more fundamental problem, namely, the kind of layperson mentality that is attracted to the biological sciences.

Most biological fields are still largely taught in a descriptive fashion, according to the naive and fallacious 'baconian' philosophy which believes that 'the facts speak for themselves'. Individuals who adopt this view of science are often endowed with a high ability for rote memorization and an insatiable appetite for manipulation of 'black-box' recipes, but with poor imagination, little reflection and almost no scepticism. The resulting phenotype is a researcher with high uncertainty avoidance behaviour (with the corresponding dose of dogmatism), a poor sense of taste for fundamentals and little recognition of the inherent ambiguity of empirical evidence — the 'kit' generation, as described by Davies and Pugsley². Of necessity, such black-box-driven technicians produce much data but little thought or ideas; any digestion is relegated to the prepackaged restriction nucleases.

Although the layman may believe that science is the straightforward accumulation and ordering of facts, the scientist who seeks a mechanistic understanding of the complexity of nature should have a more profound appreciation of the uncertainty of observations and interpretations of data. As Kuhn put it, "there are always many alternative conceptual schemes capable of bringing order to any prescribed list of observations"³; unfortunately, there seem to be fewer and fewer people in the biological sciences able to propose new schemes, or even to question the accepted ones.

Nobel Laureate Luis Leloir, who described himself as "a bad practising physician because I was never sure of the diagnosis or of the treatment"⁴, displayed the proper scepticism about his own inferences, but would have a hard time succeeding in those fields which now frown upon uncertainty and imagination.

Imagination is the mother of scepticism, for it allows one to create alternative possibilities and thus introduces uncertainty about the reality of propositions (both one's own and others'). However,

imagination is selected against in most of biology. Sir Hans Krebs relates an occasion in which one biochemist denigrated a then-new proposal by Jacques Monod as "just the sort of fantasies to which Mediterranean people are prone, but in which the British do not indulge"⁵. This is the kind of mentality that is often opposed to hypothetical thinking, and which too readily transforms palatable propositions into tenured 'facts', without imagining that observations can be interpreted in many different but testable ways, and that the inferences drawn from data are never self-evident (especially in an age when many of the 'obvious' discoveries have already been made). It is this kind of mindset which we believe is pervading the biological sciences and churning out ASTs.

JUAN P. INFANTE
VIRGINIA A. HUSZAGH

*Institute for Theoretical Biochemistry
and Molecular Biology,
PO Box 4512,
Ithaca,
New York 14852-4512, USA*

1. Rosner, J. L. *Nature* **345**, 108 (1990).
2. Davies, J. & Pugsley, A. *Trends biochem. Sci.* **15**, 137 (1990).
3. Kuhn, T. S. *The Copernican Revolution* 74 (Harvard University Press, 1957).
4. Leloir, L. F. *Ann. Rev. Biochem.* **52**, 15 (1983).
5. Krebs, H. A. in *The Creative Process in Science and Medicine* (eds Krebs, H. A. & Shelley, J. H.) 105 (Excerpta Medica, Amsterdam, 1975).

Nobel women

SIR—Sachi Sri Kantha (*Nature* **344**, 582; 1990) omits from the list of women winners of the Nobel prize, Selma Langerlöf, who won the literature prize in 1909 (*Encyclopedia Britannica*, 14th Edition, **13**, 592); I like her Gösta Berglings and children's stories.

LEONARD X. FINEGOLD

*Department of Physics,
Drexel University,
Philadelphia, Pennsylvania 19104, USA*

Mass poisoning

SIR—Slack *et al.* (*Nature* **345**, 583; 1990), writing about mass-poisoning between May 1981 and March 1983 in the Madrid area of Spain, apparently involving aniline-degraded, reprocessed rapeseed oil as the causative agent, state that the events, reported to be lethal to 340 of more than 20,000 affected individuals, "probably represent the worst recorded outbreak of food-borne chemical intoxication ever documented." However, an event in the winter of 1971–72 in Iraq, involving a methylmercury fungicide present on wheat used for bread baking, was reported lethal to some 459 individuals^{1,2}. Some 6,500 people were reported to have

been hospitalized among a much larger number intoxicated.

ROBERT A. MICHAELS

*RAM TRAC Corporation,
91 Northumberland Drive,
Schenectady, New York 12309, USA*

1. Bakir, F. *et al.* *Science* **181**, 230–41 (1973).
2. Marsh, D. O. in *The Toxicity of Methyl Mercury* (C. U. Eccles and Z. Annau, editors), Baltimore and London, Johns Hopkins Series in Environmental Toxicology, pp.45–53, 1987.

Beginning of AIDS

SIR—In a leading article (*Nature* **346**, 92; 1990), it is stated that the seaman (*sic*) who died in a Manchester hospital in 1959 of what is now believed to be AIDS had infected his wife who in turn had infected her youngest daughter. This is not true. For the record, the 25-year-old male patient who died, and who had been to sea in 1955–57, was unmarried and had no (known) offspring. There is no reason to suppose he infected anyone.

Details of his case were reported by Williams, Stretton and Leonard in 1960 (*Lancet* **ii**, 951–55).

TREVOR B. STRETTON
*Manchester Royal Infirmary,
Oxford Road, Manchester M13 9WL, UK*

SIR—Your leading article states, correctly I believe, that the rate at which AIDS spreads is determined principally "by the rate at which infected people acquire and infect new sexual partners in the interval between infection and death". But your conclusion "that the spread can be controlled only by individual precautions to ensure that sexual intercourse does not lead to infection by HIV and that other high risk behaviour is avoided" cannot be left unchallenged.

Reducing the number of sexual partners, preferably to one, reduces the rate of spread more substantially. "Till death do us part" has much to recommend it.

IAN DAVIDSON

*Holly Wood House,
Broom Way, Oatlands Park,
Weybridge, Surrey KT13 9TG, UK*

Simple words

SIR—The News item on "Genome research" (*Nature* **345**, 654; 1990) mentions *Arabidopsis thaliana* once and *Arabidopsis* three times. Is it just too much to ask that the general reader (who in the United States is being taxed for \$25 million to pay for this project) be informed that this plant happens to be a member of the Brassica or mustard family, and therefore somewhere in between the human and yeast families which are actually mentioned by names immediately recognizable by all?

H. R. CATCHPOLE

*110 West Oak Street,
Chicago, Illinois 60610, USA*

Confusion worse confounded

SIR—As the owner of a hardware store, I feel it is my duty to comment on the letter from C. H. Evans (*Nature* 345, 658; 1990). The writer waxes sentimental about the British 'system' of weights and measures to which the United States alone so obdurately clings.

Closer examination reveals that we don't have a system, we have a patchwork quilt of systems; systems whose units cannot be added, subtracted, multiplied or divided with ease, and hardly anyone knows how to use them. Learning a new system would come as a welcome relief to those who have actually learned the 'British system' and have to use it for complex operations.

The other day, a customer asked for a piece of lumber cut to "five feet two and a half inches and one of those little marks" (a sixteenth). What could be more elegant? A carpenter more familiar with the system could translate that to more manageable sixty-two and nine-sixteenths inches. Of course if he has to add the width of a "one-by-twelve" ($\frac{3}{4}$ " $\times$ $11\frac{1}{4}$ ") and deduct the thickness of a "two by four" ($1\frac{1}{2}$ " $\times$ $3\frac{1}{2}$ "), he figures $62\frac{9}{16} + 11\frac{1}{4} - 1\frac{1}{2} = 72\frac{5}{16}$. In building a house (or a space shuttle), thousands of these tedious computations are carried out and each one is a potential source of error.

We start to see that within the 'system', things are not what they say they are. Two-by-fours are not 2×4 and they haven't been for years. The two-by-fours in a hundred-year-old house are 2×4 , but the two-by-fours in a fifty-year-old house are $1\frac{5}{8} \times 3\frac{5}{8}$, and in a new house they are $1\frac{1}{2} \times 3\frac{1}{2}$. Half-inch galvanized pipe isn't half an inch anywhere. The inside diameter is about $\frac{5}{8}$ " and the outside diameter is about $1\frac{1}{16}$ ". Plumbers know what size to ask for, but most others make the mistake of trying to measure the pipe and become hopelessly confused.

Electrical wire comes in gauges. As the wire gets bigger, the gauge number gets smaller. 12 ga. wire will conduct $\frac{2}{3}$ as much current as 14 ga.; 10 ga. conducts $\frac{3}{2}$ as much current as 12. Crystal clear! Nuts, bolts and wood screws also have gauges. Of course now as the bolt gets bigger, the gauge number gets bigger. What could be simpler than nails? Nails are measured in pennies. The symbol is 'd' as in 'penny'. A 4d nail is $1\frac{1}{2}$ " long. 6d is 2", 8d is $2\frac{1}{2}$ ", 10d is 3". So, it should be perfectly obvious that a $3\frac{1}{2}$ " nail will be . . . that's right, 16d.

Drill bits cover all bases. There are of course fractional bits in increments of $\frac{1}{64}$ " where it is immediately obvious that $\frac{25}{64}$ is larger than $\frac{3}{8}$ but smaller than $\frac{13}{32}$. Among the interstices between fractions there are number drills, an inverse system with no. 1 a little smaller than $\frac{1}{4}$ " going

"down" to no. 80 a little larger than a hair. Also interspersed between fractions are the letter drills irregularly spaced from A to Z with A a little larger than a no. 1 going up to Z smaller than $\frac{1}{2}$ ".

Concrete comes by the cubic yard, lumber by the board foot, shingles by the square, yarn by the skein, but a sack of cement is always 94 pounds. The tape in your walkman travels at $1\frac{7}{8}$ inches per second, which adds up to quite a few furlongs per fortnight. It's a Jim Dandy system all right, and any country that would give it up for something as straight forward as metric has no sense of humour.

ERNEST L. ASTEN

*Cliff's Variety,
479 Castro Street,
San Francisco, California 94114, USA*

Some good news

SIR—Seth Shulman, on the basis of a Carnegie Foundation report about nuclear smuggling, writes that the 1980s were a bad decade for nuclear proliferation ("Seven more nations nearly 'nuclear'", *Nature* 345, 4; 1990). The seven listed are Argentina, Brazil, India, Iraq, North Korea, Pakistan and South Africa.

Shulman's summary may be accurate, but the picture presented is unduly alarmist. In fact there was substantial progress in stemming proliferation during the 1980s (at the start of which I was assistant director general of the International Atomic Energy Agency).

Ten years ago, Argentina and Brazil seemed to be racing each other to be the first to carry out a nuclear test. Since the advent of democratic governments, each has undertaken several measures to promote confidence in the other's nuclear activities, and they are now cooperating in joint ventures. The Brazilian constitution now explicitly requires that all Brazil's nuclear activities be exclusively peaceful.

South Africa, far from being a "*de facto* nuclear power", is considering accession to the Non-Proliferation Treaty (which 27 nations, including Spain, Egypt, Turkey and Saudi Arabia, joined during the 1980s).

The danger in India and Pakistan is hardly a phenomenon of the 1980s. India tested in 1974, and several years earlier Zulfikar Bhutto declared that Pakistan would eat grass, if necessary, to catch up with India.

Because of the understandable suspicions about Iraq, Saddam Hussein has been unable to replace the large Tammuz reactor the Israelis destroyed in 1981 and thereby put an end to any plans Iraq might have had to take the plutonium route to the bomb. Besides the flurry about

smuggled capacitors, there have been reports that Iraq is now trying to acquire enrichment technology. They are as yet unsubstantiated and the general impression is that Iraq will give priority to developing its chemical warfare capabilities in response to Israel's nuclear arsenal.

The only new risk in the 1980s stems from North Korea. Disquieting though the reports about its actions may be, they are at least offset in the global balance by the improvement in nuclear relations between Argentina and Brazil and South Africa's retreat from the threshold.

Smuggling of nuclear technology has certainly reached disturbing proportions, but this is partly because exports that were legal in the 1960s and 1970s are (rightly) against the law today.

DAVID FISCHER

*15 Willow Walk,
Cambridge CB1 1LA, UK*

A woman's place

SIR—Reviewing Michael H. Brown's book *The Search for Eve* (*Nature* 345, 395; 1990), J. S. Jones mentions the idea that the spread of one mother's genes accompanied the origin of language and thus, in M. H. Brown's words, "woman had the first word". It is surprising that this obviously wrong idea is surfacing again after having been buried before 1305 by Dante, who wrote in *De vulgari eloquentia* (I, IV, 3; my translation): "... while in the scriptures you find that the first to speak was the woman, it seems more rational to think that it was the man, because it is indeed inconsistent to assume that such an important human activity could have possibly derived from a woman".

MARCO FRACCARO

*Collegio Cairoli,
I-27100 Pavia, Italy*

God and science

SIR—Scientists crusading against religion are sometimes inclined to throw out data points that do not fit a preconceived curve. Various kinds of fanaticism and obscurantism are given as examples of the bad effects of belief: instances of the relief of suffering, or resistance to oppression, are ignored. A causal link is assumed in the one case, but not in the other.

I applaud Christopher Lote's suggestion of an opinion poll on the religious beliefs of your readers, provided of course that it is designed well enough for the results to mean something. For example, if I simply say that I am a Christian, that tells no-one whether I am some kind of creationist, or whether I accept both Christianity and Darwinism as bases for further thought.

A. J. BUNTING

*Institute of Oceanographic Sciences,
Deacon Laboratory, Wormley, Surrey, UK*

Risky arguments over cause and effect

Statistical analyses of public health risks often cause general confusion, but that may be because the experts do not always tell the whole story.

THE person who complains mightily against nuclear power but insists on the right not to wear a motorcycle helmet typifies, for those with a logical cast of mind, a common inability to grasp the nature of statistical arguments. Risk assessment has become a recognized branch of actuarial science, and those who have gone to great lengths to establish the relative probabilities of death from radioactive fall-out, smoking, falling off ladders and driving to the supermarket would naturally prefer that the general public should order its political and social priorities according to their calculations.

But things are rarely this tidy. Recent weeks have seen the re-emergence in the United States of three issues which revolve around the actuarial assessment of a possible health risk, and which have caused some scientists to sigh once more that statistical ideas are insufficiently understood. Two months ago, to the chagrin of many biologists and most physicists, the Environmental Protection Agency (EPA) decided that the possibility of electromagnetic radiation from power lines causing certain cancers deserved a rigorous assessment.

Then, two weeks ago, the EPA pleased some scientists but got into trouble with environmentalists for circulating the idea that low-level radioactive waste, such as hospitals produce, does not deserve to be tarred with the same brush as high-level waste from nuclear power stations. On the grounds that the actual dangers the waste presents is quite small, EPA suggested that it could be disposed of in less stringently controlled ways.

Finally, organizations of US veterans from the Vietnam war last week sued the Veterans Administration and the Centers for Disease Control (CDC) to restart studies of ill health and disease in veterans caused by exposure to the defoliant Agent Orange, studies which were started in 1979 but abandoned four years ago because the CDC failed to find any groups of soldiers that had demonstrably received large doses of dioxin (see page 498). These issues have no particular political connection — indeed the EPA seems to be 'green' on the question of electromagnetic radiation, but 'pronuclear' on low-level radioactivity — but in each case the scientific argument turns on the reality of health risks which, by anyone's account, are not large. The idea that 60-Hz electromagnetic radiation might cause cancer has

long been ridiculed by physicists, principally because they cannot imagine any mechanism that would connect the two phenomena. But some epidemiological studies published in one or two reputable journals have continued to find excess leukaemias and brain cancers in children living near power transmission lines (see *Nature* **345**, 463; 1990).

The Agent Orange case is rather different. Dioxin, a constituent of the defoliant, is a powerful carcinogen in animals, although its toxicity in humans is hotly debated. Any Vietnam veteran who was in or near some part of the jungle over which Agent Orange was sprayed and who, many years later, contracted cancer, can hardly be blamed for thinking that cause and effect are at work.

But efforts to find such a link have proved unsuccessful on two counts. A minimal requirement for such a study is that soldiers can be divided into those who received doses of dioxin and those who did not; otherwise, even if health anomalies turn up, the extent to which Agent Orange might be blamed is impossible to determine. The CDC, working with military records, say they cannot find credible evidence that any significant number of soldiers were ever close enough to the site of spraying to have received any measurable exposure to dioxin.

More fundamental still, a study by the Veterans Administration itself of the post-war deaths of 50,000 Vietnam veterans failed to turn up any unusual or excessive causes of death that might be attributed to dioxin contamination. The only anomalously high death rate was due to non-Hodgkins lymphoma — but that was among Navy personnel who were certainly never exposed to Agent Orange.

To the actuarially minded scientist, this is a neat conclusion.

Dioxin is a dangerous substance, but an examination of people who might have been exposed to it shows no signs of that danger having materialized. Similar logic is behind the EPA's suggestion that radioactive wastes which would give a person a dose of less than 10 mRem per year need not really be called radioactive, because the health risk they pose is negligible in comparison to those from other (including natural) sources of radioactivity.

Pleading in a purely scientific court, the actuary might argue for dismissal of the health risks in all these cases. The case against electromagnetic radiation should

be dismissed for lack of evidence; radioactivity and dioxin are both known felons but should nevertheless be judged not guilty of these particular charges.

But only in the case against Agent Orange does the actuarial verdict hold up to examination. Agent Orange was applied in finite quantities to a known population; that population having been studied, no ill effects were found. The conclusion is not that dioxin is harmless, but that in this instance it caused no detectable harm.

The argument for 'declassifying' low-level radioactive waste is misleadingly similar in appearance but fundamentally different in form. The dangers presented by low-level radioactivity must be assumed to apply to an infinite population (because the waste is being continuously generated). If actuarial arguments mean anything, they mean in this case that an additional risk applied to an unrestricted population means additional deaths. The balancing of risks against the cost of suppressing them demands a political rather than a scientific judgement. But even neglecting politics it is hard to argue in favour of adding to the environment, out of a sense of mathematical virtue, a risk that was previously guarded against.

As for the supposed dangers of electromagnetic radiation, there is patently insufficient evidence, statistical or otherwise, on which to base a rational decision. Those who find it objectionable that the EPA is even considering the subject seem to see the issue as quackery invading what ought to be a scientific agency. But the EPA exists in the first place to assuage public fears, not to do research in basic science; the perception of a public health problem merits its attention as much as one that can be measured. In trying to get to the bottom of a long-standing controversy, the EPA is simply carrying out its purpose.

Translating statistical analyses into public health policy would be easier if the nature of the central arguments were more widely understood. But in only one of these three cases are the simple-minded actuarial results enough to support an acceptable public policy. Before they berate others for not understanding the methods of statistical inference, scientists with strong opinions should check that their own more subtle understanding of the process is not being used to disguise prejudice.

David Lindley

Planning for serendipity

Solomon H. Snyder

MARIJUANA is one of the most intriguing of all drugs, used since antiquity both for medicinal and recreational purposes. Its psychoactive ingredient, delta-9-tetrahydrocannabinol (Δ^9 -THC) is notably potent, altering human behaviour at doses of only a few micrograms per kilogram. Moreover, it acts stereospecifically, virtually ensuring the existence of selective receptors. Accordingly, when opiate and other drug and neurotransmitter receptor-binding sites were first identified in the mammalian brain 17 years ago, pharmacologists expected Δ^9 -THC receptors to follow soon thereafter, but were disappointed. The elegant molecular cloning of a pharmacologically relevant Δ^9 -THC receptor reported by Matsuda *et al.* on page 561 of this issue¹ should now focus attention on this important class of drugs.

For many years, therapeutic effects in cases of epilepsy, asthma, nausea, pain and hypertension have been documented both for marijuana-plant extracts and purified cannabinoids². By the late 1930s, the chemical structure of Δ^9 -THC was sufficiently well known for drug companies to be able to synthesize analogues in the hope of retaining therapeutic effects but eliminating psychoactivity. By World War II, the draconian provisions of the US 1937 Marijuana Tax Act virtually terminated all research on the drug, research that remained dormant until Mechoulam's demonstration³ in 1964 of the correct structure of Δ^9 -THC. In the ensuing two decades, extraordinarily potent, Δ^9 -THC derivatives have emerged, some of which display pharmacological selectivity.

With such potent agents, one would have anticipated abundant results from research on the receptors, but the lipophilicity of Δ^9 -THC-related ligands hindered progress. Recently, Howlett and associates have had success both in binding experiments⁴ and in investigating second-messenger candidates⁵. These authors observed limited (30–40 per cent) inhibition of adenylyl cyclase in brain tissue and cell culture systems by cannabinoids with structure-activity relationships that fit known cannabinoid pharmacology. By using tritiated samples of the potent cannabinoid CP-55940, they observed receptor binding with the appropriate pharmacology, albeit with

substantial non-specific binding.

Final success in cloning a cannabinoid receptor, however, did not stem from a direct interest in marijuana. Instead, Matsuda *et al.*¹ used a strategy of planned serendipity to seek novel G-protein-linked receptors using oligonucleotide probes derived from known receptors, in this case a receptor for bovine substance K. They cloned a complementary DNA whose sequence did not resemble any known protein. With seven hydrophobic

potencies closely resembling their known effects in brain tissue and neural cells in culture. But other known neurotransmitters and peptides fail to alter adenylyl cyclase in the transfected cells.

A direct assault on important questions regarding Δ^9 -THC may now be possible. What is the receptor's normal function? Is there an endogenous ligand? Some hints come from *in situ* hybridization studies¹. In rat brain, messenger RNA encoding the receptor is highly concentrated in parts of the cerebellum, hippocampus and the cerebral cortex as well as other areas, closing mapping the localization of CP-55940 binding sites⁶, which in turn closely resembles the distribution

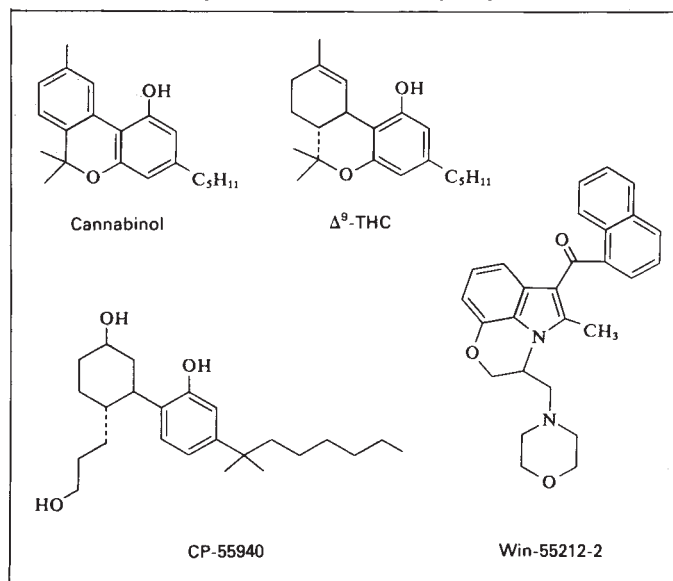
of the inositol triphosphate receptor⁷. Could the Δ^9 -THC receptor be linked to the phosphoinositide second-messenger system? Perhaps the inhibition of adenylyl cyclase by cannabinoids relates to phosphoinositide turnover. For instance, dopamine and serotonin can inhibit phosphoinositide turnover in some instances, decreasing intracellular calcium release, which may lower activity of the largely calcium-dependent brain adenylyl cyclase⁸. Decreases in phosphoinositide turnover in response to Δ^9 -THC have been reported⁹.

Robust expression of the Δ^9 -THC receptor in transfected cells should provide much stronger signals than previously available both in second-messenger and ligand-binding research. It may then be possible to search more efficiently for Δ^9 -THC antagonists, endogenous ligands and mechanisms whereby receptors are coupled to second messengers.

Because the maximal reduction of adenylyl cyclase by Δ^9 -THC is not as great as that by CP-55940 in cells with expressed receptors, Δ^9 -THC itself may behave as a mixed agonist-antagonist.

The diverse pharmacological actions of THC-derivatives implies the existence of receptor subtypes: cannabinoid receptor cDNA could be used to search for other members of the postulated receptor family. If the receptors with the potential for mediating the therapeutic uses of cannabinoids are different from those responsible for their psychoactive effects, the cloning work should vastly accelerate drug development, fulfilling the ancient promise of marijuana as medicine. □

Solomon H. Snyder is in the Department of Neuroscience, Johns Hopkins University School of Medicine, 725 North Wolfe Street, Baltimore, Maryland 21205, USA.



Cannabinoid structures. Δ^9 -THC is the psychoactive constituent of marijuana. Strict structure-activity relationships implying a selective receptor are evident in the complete loss of psychoactivity with the modest chemical alterations of cannabinol. CP-55940 is substantially more potent as an analgesic than Δ^9 -THC and has been employed as a tritiated ligand to label marijuana receptors^{4,6}. Win-55212-2 was first synthesized as a potent analgesic related to nonsteroidal anti-inflammatory agents (NSAIDs) and only subsequently discovered to bind with high affinity to marijuana receptors¹⁰.

domains, similarity to several G-protein-coupled receptors and a relative molecular mass of about 53,000, it fitted properties expected of G-protein-associated receptors. In cultured CHO-K cells transfected with the cDNA, cannabinoids inhibit adenylyl cyclase with relative

1. Matsuda, L.A., Lolait, S.J., Brownstein, M.J., Young, A.C. & Bonner, T.J. *Nature* **346**, 561–564 (1990).
2. Snyder, S.H. *Uses of Marijuana* (Oxford, New York, 1971).
3. Gaoni, Y. & Mechoulam, R. *J. Med. Soc.* **86**, 1646 (1964).
4. Devane, W.A., Dysarz, F.A., Johnson, M.R., Melvin, L.S. & Howlett, A.C. *Mol. Pharmacol.* **36**, 605–613 (1988).
5. Howlett, A.C., Johnson, M.R., Melvin, L.S. & Milne, G.M. *Mol. Pharmacol.* **33**, 297–302 (1988).
6. Herkenham, H. *et al.*, *Proc. natn. Acad. Sci. U.S.A.* **87**, 1932–1936 (1990).
7. Worley, P.F., Baraban, J.M. & Snyder, S.H. *J. Neurosci.* **9**, 339–346 (1989).
8. Chuang, D.M. *A. Rev. Pharmac. Tox.* **29**, 71–110 (1989).
9. Chaudry, A., Thompson, R.H., Rubin, R.P. & Laycheck, S.G. *Mol. Pharmacol.* **34**, 543–548 (1988).
10. Ward, S.J. *et al.* *Proceeding of Committee on Problems of Drug Dependence* (US Govt Printing Office, 1990).

Spin-off for solid-state studies

Jacek Klinowski

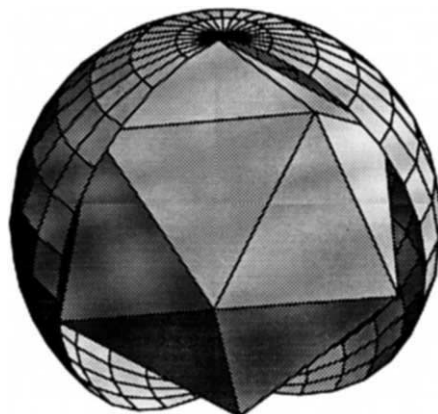
EXPERIMENTS reported by Wu *et al.* on page 550 of this issue¹ have resulted in the best-resolved ^{27}Al nuclear magnetic resonance (NMR) spectra yet measured in the solid state, bringing together recent developments in synthetic inorganic chemistry, catalysis, physics, group theory and crystallography and opening up exciting perspectives for the future. The authors have studied the industrially important aluminophosphate zeolite VPI-5, a molecular sieve containing channels which comprise 18-membered rings of tetrahedrally coordinated aluminium and phosphorus atoms, using the NMR technique of double rotation recently developed by Pines (one of the present authors) and colleagues. The results demonstrate the ability of this technique to yield unique structural information on this mineralogically important element.

Wilson *et al.*² discovered in 1982 that under certain conditions aluminium phosphate can form open crystalline structures containing systems of channels and cavities of molecular dimensions, similar to those of aluminosilicate zeolites. The AlPO_4 molecular sieves are built from alternating AlO_4 and PO_4 tetrahedra. Some of them have the framework topologies of known zeolites, but many have novel structures. AlPO_4 sieves are synthesized from gels containing aluminium, phosphorus and at least one organic structure-directing template. Incorporation of a silicon source into an aluminophosphate gel results in the formation of silicoaluminophosphates (SAPO), and the incorporation of a metal M (such as Mg, Mn, Fe, Co or Zn) into AlPO_4 and SAPO gives the MAPO and MAPSO sieves respectively³. Some of these have high Brønsted acidities and thus a considerable potential to act as catalysts as well as media for ion exchange and molecular separation.

Until recently the largest channels in any molecular sieve were based on 12-membered rings of tetrahedral atoms and were invariably less than 10 Å wide. In 1984 Smith and Dytrych⁴ described a hypothetical series of molecular sieves, one of which had channels 12 Å in diameter. The article aroused little interest, however, until Davis *et al.*⁵ described the preparation of a crystalline aluminophosphate, VPI-5, with the precise structure predicted by Smith and Dytrych. The new development caused much excitement, especially as related SAPO and MAPO materials could also be prepared. They have possible applications in the pharmaceutical industry for the purification of large molecules and in petroleum refining, where they could be used to crack very large hydrocarbons which are

at present discarded as bottom-of-the-barrel residue. VPI-5 can be hydrated reversibly, and this is the process that Wu *et al.*¹ have now examined.

NMR spectra cannot normally be measured in solids in the same way that they are obtained routinely in liquids. This is because of the presence in solids of net anisotropic interactions, which in liquids are averaged by the rapid thermal molecular motions. Although molecular motions in certain solids are sufficiently unconstrained for NMR spectra to be obtained



Euclid's last problem — inscribing an icosahedron in a sphere: a starting point for ^{27}Al NMR. without resorting to special techniques, in general conventional NMR of the solid state yields not sharp spectral lines but a broad hump which conceals most of the interesting information. Individual lines corresponding to nuclei of the same species in different magnetic environments, for example, cannot be resolved.

Andrew *et al.*⁶ and Lowe⁷ came up with a partial solution to this problem. They showed that when powdered samples of many solids are spun rapidly about an axis inclined at an angle of $54^\circ 44' 8''$ (the so-called 'magic angle') to the direction of the magnetic field, the resolution of the spectrum improves dramatically: the broad line is reduced to a narrow peak. Magic-angle spinning (MAS) imposes an average axial symmetry on an otherwise asymmetric environment. The magic angle (at which the second-order Legendre polynomial $3\cos^2\theta - 1$ equals zero) is the angle between the body diagonal of a cube and each of its faces. The most common magnetic interactions, such as the chemical-shift anisotropy and dipole-dipole coupling, can all be represented in terms of second-rank spherical harmonics, so to average these interactions we do not need to move the sample over a complete sphere (as occurs naturally in liquids), but only to impose cubic symmetry by spinning around the magic angle.

MAS works very well with spin-half

nuclei such as ^{13}C , ^{15}N , ^{29}Si and ^{31}P , but is less successful with the so-called quadrupolar nuclei, such as ^{11}B , ^{17}O , ^{23}Na and ^{27}Al , which have an asymmetric distribution of nuclear charge. Although MAS does reduce the linewidth of NMR spectra for the latter nuclei, this is often insufficient for the spectra to be fully interpreted. In particular, ^{27}Al MAS NMR in solids has in the past been something of a disappointment in comparison with, for example, ^{29}Si , which gives well resolved MAS spectra. This shortcoming is unfortunate because aluminium is the second most abundant element in the Earth's crust, and over half of all known minerals are silicates and aluminosilicates. To obtain high-resolution spectra of this nucleus is therefore a highly worthwhile goal; so the success of Wu *et al.*¹ will come as welcome news to many chemists, mineralogists, metallurgists and materials scientists.

Quadrupolar nuclei interact not only with the magnetic field in which the sample is immersed but also with the electric-field gradient; the combination of both effects results in an anisotropy that can no longer be removed by magic-angle spinning alone. Group theory indicates that the quadrupolar interaction transforms as a fourth-rank spherical harmonic, and is averaged by motion corresponding to icosahedral symmetry. The icosahedron is a better approximation to a sphere than is a cube (see figure), and the task of inscribing an icosahedron inside a sphere is the famous 'Euclid's last problem' (A. Mackay, personal communication). Icosahedral symmetry cannot be achieved by rotating the sample about a single axis⁸. Pines and his group, together with Samoson and Lippmaa, realized that there were two different ways of implementing the symmetry of an icosahedron. One, which they call dynamic angle spinning (DAS), is to rotate the sample sequentially about two different axes, inclined to the magnetic field at angles of 37.38° and 79.19° : the sample is rotated about the first axis, reoriented, rotated about the second axis and so on. DAS NMR probeheads are already commercially available. The other possibility is double rotation, in which the sample is spun simultaneously about two axes, the first inclined to the magnetic field at the magic angle and the second at the angle given by the zero of the fourth-order Legendre polynomial. This polynomial is $35\cos^4\theta - 30\cos^2\theta + 3$, one of the solutions being 30.6° .

To achieve double rotation in practice is an awesome engineering task. The spinning rotors are driven by a flow of gas, which must be delivered to one rotor embedded in another. And if the inner rotor (which contains the sample) is to spin freely, its net angular momentum must lie exactly along the axis of the outer

rotor. The first DOR experiments in 1988 showed that the widths of the peaks are greatly reduced in comparison with MAS, being little greater than those measured in liquids. The greater spectral resolution is accompanied by an improvement in sensitivity (that is, the NMR-active nuclei can be more dilute). It is interesting to note (A. Mackay, personal communication) that the principle of DOR is similar to that of the Gandolfi camera^{9,10}, used by crystallographers to obtain X-ray powder patterns of very small single crystals, which also rotates about two independent axes. There is also an intriguing link between DAS/DOR and the icosahedral quasi-crystals predicted by Mackay¹¹ and subsequently prepared^{12,13}.

DAS and DOR have considerable potential for the study of minerals, heterogeneous catalysts, zeolites, glasses, polymers and superconductors. For example, the ¹⁷O DOR NMR spectrum of diopside¹⁴ clearly reveals the three crystallographically inequivalent oxygen sites in this mineral. Wu *et al.*, using Pine's apparatus, now provide the important extension of DOR to ²⁷Al. Whereas the conventional ²⁷Al MAS NMR spectra of VPI-5 feature a broad single signal corresponding to tetrahedrally coordinated aluminium, the DOR spectra contain a wealth of structural information. We learn that, although the structure of VPI-5 calls for only two crystallographically distinct sites for Al (or P), hydration of the material creates further structural inequivalence. The various inequivalent aluminium atoms in the structure (as many as eight can be monitored simultaneously in these astonishingly well-resolved spectra) evidently differ in their affinity for water. Furthermore, unlike the ¹⁷O and ²³Na DOR spectra, there are no spinning sidebands, because of the relatively small anisotropy involved. And last but not least, the results provide interesting information on the chemical environment of water inside the molecular sieve. □

Jacek Klinowski is in the Department of Chemistry, University of Cambridge, Lensfield Road, Cambridge CB2 1EW, UK.

Antibiotics cure asexuality

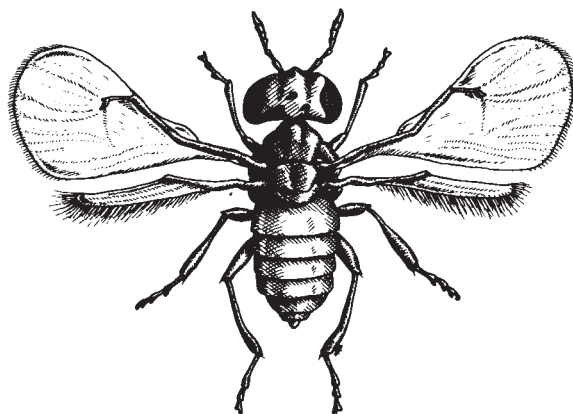
Laurence D. Hurst, H. C. J. Godfray and Paul H. Harvey

EGGS contribute cytoplasm to embryos and sperm do not. Extra-nuclear genetic material in males is therefore at an evolutionary dead end. As a consequence, genes in the cytoplasm that cause an egg or embryo to become female will be favoured by selection; for example mitochondrial DNA that finds itself in a male will not be passed on to the next generation, and any mutant in that DNA which causes a mammalian egg to be fertilized by an X-bearing sperm will be favoured, until countered by modifiers on nuclear genes which favour a 50:50 sex ratio. Cytoplasmic elements bias sex ratios through several mechanisms¹⁻⁴. Now, Stouthamer,

strains do. If very small doses of antibiotic are prescribed, or if the temperature is lowered, then the strains revert to asexual reproduction. But, if antibiotic treatment is continued over several generations then the population becomes permanently sexual and does not revert after cessation of treatment.

One likely explanation for these results, as previously suggested by Legner⁶, is that the expression of asexuality is controlled by a cytoplasmically inherited microorganism. This microorganism is destroyed by antibiotics and either destroyed or inhibited by high temperatures. Evidence for the cytoplasmic inheritance of

David Nash
asexuality and sex change also comes from breeding experiments in which sexual females, caught in the wild, are crossed with males derived from asexual strains through heat treatment. The strains remain sexual even when, after many generations of backcrossing, most of the nuclear genome is derived from asexual strains. Stronger evidence for the influence of a microorganism would require the identification of an organism present in asexual individuals that is absent from sexual females. The



Parasitoid wasp of the genus *Trichogramma* — the wasp parasitizes the eggs of the ant-tended lycaenid butterfly, *Jaimeus evagoras*.

Luck and Hamilton⁵ reveal another — extraordinary — one. A genetic factor in the cytoplasm of some female wasps converts presumptive male eggs into female ones; what is more, the genetic factor may be a microorganism because, after treatment with antibiotics, male-to-female sex change can be 'cured' and the females produce males.

Viable populations from which males are absent must reproduce asexually. Such asexual reproduction is reasonably common in the Hymenoptera, the group that contains the social ants, bees and wasps, as well as non-social wasps, parasitoid wasps and saw flies. Parasitoids of the genus *Trichogramma* are minute wasps (about 1 mm in length) that parasitize the eggs of other insects, particularly moths (see figure). A large number of *Trichogramma* species are known, some of which reproduce wholly sexually or asexually whereas others are composed of mixtures of sexual and asexual strains. Stouthamer and colleagues⁵ discovered that if females of asexual species or strains are allowed to feed on honey containing certain antibiotics, or are kept at elevated temperatures (over 30 °C), they begin to produce males and females just as sexual

spectrum of antibiotics that can cause a switch in reproductive behaviour suggests that the organism is a prokaryote. Stouthamer and his colleagues at the University of Rochester say that their preliminary, unpublished studies have revealed a candidate bacterium which may be the causal agent.

Is asexual reproduction induced by microorganisms a curiosity of wasps of the genus *Trichogramma*, or is it more widespread? There are numerous reports of asexual populations of various hymenopterans in both laboratory and field being rendered sexual by exposure to high temperatures. Subsequent exposure to milder temperatures typically allows reversion to asexuality⁷. According to Stouthamer (personal communication), when asexuality is curable by high temperature, it is also curable by antibiotics; and, importantly, when organisms maintain asexuality at high temperature, antibiotic treatment is equally ineffective. This is circumstantial evidence but it does point to the phenomenon being widespread within the group.

Asexuality induced by microbes has been suspected amongst other groups. *Lecanium cerasifex*, a soft-scale homop-

1. Wu, Y. *et al.* *Nature* **345**, 550–554 (1990).
2. Wilson, S.T., Lok, B.M., Messina, C.A., Cannan, T.R. & Flanigen, E.M. *J. Am. chem. Soc.* **104**, 1146–1147 (1982).
3. Flanigen, E.M., Lok, B.M., Patton, R.L. & Wilson, S.T. *Pure & appl. Chem.* **58**, 1351–1358 (1986).
4. Smith, J.V. & Dytrych, W.J. *Nature* **309**, 607–608 (1984).
5. Davis, M.E., Saldarriaga, C., Montes, C., Garcés, J. & Crowder, C. *Nature* **331**, 698–699 (1988).
6. Andrew, E.R., Bradbury, A. & Eades, R.G. *Nature* **182**, 1659 (1958); **183**, 1802–1803 (1959).
7. Lowe, I.J. *Phys. Rev. Lett.* **2**, 285–287 (1959).
8. Samoson, A., Lippmaa, E. & Pines, A. *Mol. Phys.* **65**, 1013–1018 (1988).
9. Gandolfi, G. *Miner. Petrol. Acta* **13**, 67–74 (1967).
10. Graeber, E.J. & Jelinek, D.A. *Norelco Rep.* **13**, 91–92 (1966).
11. Mackay, A.L. *Sov. Phys. (Cryst.)* **26**, 517–522 (1981).
12. Shechtman, D., Blech, I., Gratias, D. & Cahn, J.W. *Phys. Rev. Lett.* **53**, 1951–1953 (1984).
13. Levine, D. & Steinhardt, P.J. *Phys. Rev. Lett.* **53**, 2477–2480 (1984).
14. Chmelka, B.F. *et al.* *Nature* **339**, 42–43 (1989).

ATMOSPHERIC TRACE GASES

teran insect, has asexual and sexual strains. Asexual organisms can be distinguished from their sexual relatives by the presence of a vertically transmitted needle-like bacterium found only in asexuals⁷. Unlike the *Trichogramma* case, however, it is not known whether removal of this bacterium allows reversion to sexuality. Asexual development in turkeys has been linked with the vertical transmission of live viruses⁸, though curiously the offspring are male. Sex change forced by cytoplasmic microbes has been demonstrated in crustaceans, where XY-presumptive males are converted into females. Fully asexual populations cannot evolve in such instances because eggs need to be fertilized by male sperm in order to develop¹. In the trichogramids, however, male wasps develop from unfertilized eggs. The sex-changed eggs likewise develop without the need for copulation. Infected wasps can thus reproduce asexually in the absence of males by laying unfertilized eggs which develop into females. Because infected females produce no males, a population of asexual wasps might be expected to develop.

It is unclear in the proposed instances of microbe-induced asexuality whether the microorganism is a manipulator or simply a mechanism. To put it another way, if all organisms could be asexual, would it be to their advantage? If the answer is yes, then by maximizing its own fitness the symbiont is also maximizing its host's fitness. If however the host's best interests are served by being sexual, then the microorganism is manipulating the host. A major problem in evolutionary biology is the identification of the selective forces maintaining sexual reproduction⁹, and one way of tackling it has been to look for ecological or genetic correlates of organisms that have abandoned sex to reproduce asexually¹⁰. But if asexuality is indeed due to the manipulation of a host by its cytoplasm, that enterprise might be rendered unprofitable. □

Laurence D. Hurst and Paul H. Harvey are in the Department of Zoology, University of Oxford, South Parks Road, Oxford OX1 3PS, UK. H. C. J. Godfray is in the Department of Pure and Applied Biology, Imperial College, Silwood Park, Ascot, Berkshire SL5 7PY, UK.

1. Legrand, J.J., Legrand-Hamelin, E. & Juchault, P. *Biol. Rev.* **62**, 439–470 (1987).
2. Skinner, S.W. *Science* **215**, 1133–1134 (1982).
3. Skinner, S.W. *Genetics* **109**, 745–759 (1985).
4. Frank, S.A. *Am. Nat.* **133**, 345–376 (1989).
5. Stouthamer, R., Luck, R.F. & Hamilton, W.D. *Proc. natn. Acad. Sci. U.S.A.* **87**, 2424–2427 (1990).
6. Legner, E.F. *Bull. Soc. Vector. Ecol.* **12**, 528–533 (1987).
7. Nur, U. *Chromosoma* **39**, 381–401 (1972).
8. Olsen, M.W. & Buss, E.G. *Genetics* **56**, 727–732 (1967).
9. Michod, R.E. & Levin, B.R. *The Evolution of Sex: An Examination of Current Ideas* (Sinauer, Sunderland, 1988).
10. Bell, G. *The Masterpiece of Nature: The Evolution and Genetics of Sexuality* (Croom Helm, London, 1982).

Burning trees and bridges

Joel S. Levine

ON page 552 of this issue¹, Lobert *et al.* identify a new and apparently important component of the biogeochemical cycle of nitrogen — pyrodenitrification, the release of N₂ by biomass burning. This process may constitute an increasingly important mechanism for returning biologically fixed nitrogen to the atmosphere, in the form of N₂ and nitrogen-containing trace gases.

In their laboratory studies, Lobert *et al.* have determined the proportions of nitro-

fixation rate (about 139 Tg yr⁻¹). Nitrogen fixation — the chemical transformation of N₂ into other nitrogen compounds such as NH₃ and NO — can be carried out by a number of microorganisms, either free-living or in association with certain plants. Industrial processes may fix about 45 Tg yr⁻¹ (ref. 2), mainly via the Haber process (in which a mixture of N₂ and H₂ is combined to form ammonia). Most industrially fixed nitrogen goes towards the production of fertilizer (in the form of ammonium

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Burning the Brazilian rainforest — upsetting the global balance of the nitrogen cycle?

gen-containing gases produced relative to the nitrogen content of the pyrolysed biomass fuel: nitrogen oxides (NO and NO₂, denoted as NO_x) account for 12 per cent, ammonia for about 4 per cent, hydrogen cyanide about 2.3 per cent and acetonitrile (CH₃CN) about 1 per cent. Very minor contributions were found from other nitrogen-containing compounds. But this adds up to only about 20 per cent of the original nitrogen content of the fuel. The authors assume that another 20 per cent is contained in organic nitrogen compounds (a very generous estimate), which were not measured in the experiment. This leaves more than half of the nitrogen in the fuel unaccounted for.

Lobert *et al.* suggest that the missing nitrogen is released in the form of N₂. Thus they estimate that production and release of N₂ through global biomass burning could represent an annual loss from the biosphere (to the atmosphere) of 12–28 × 10⁹ kg (12–28 Tg) of nitrogen as a result of tropical burning. This is equivalent to about 9–20 per cent of the estimated global terrestrial natural nitrogen

nitrate). A small amount of nitrogen (less than 10 Tg yr⁻¹) is fixed into NO by the action of atmospheric lightning³. But the bulk of atmospheric nitrogen is fixed by biological processes, and this fixation is believed to be balanced by microbial denitrification, a process in which certain bacteria reduce nitrate to N₂, N₂O or NO, with N₂ being the main gaseous product². Microbial denitrification has been thought to be the only significant denitrification process; but now Lobert *et al.* are suggesting that pyrodenitrification must also be considered.

Global biomass burning includes the burning of forests (tropical, temperate and boreal; see figure) for clearing of savanna grasslands, agricultural waste and stubble, and of wood for fuel. Paul Crutzen first identified biomass burning as a significant global source of trace atmospheric gases more than a decade ago⁴, and a conference was held recently* to assess and quantify its role and importance as a global source of radiatively active gases

* Global Biomass Burning: Atmospheric Climate and Biospheric Implications, Williamsburg, Virginia, 19–23 March 1990.

(CO₂, CH₄ and N₂O) and chemically active gases (for example CO, NO_x, non-methane hydrocarbons and H₂). M.O. Andreae (Max-Planck-Institut für Chemie, Mainz) estimated that about 8,700 Tg of dry biomass matter are burned each year, releasing 3,940 Tg of carbon to the atmosphere, mostly in the form of CO₂ (3,500 Tg C yr⁻¹) and CO (350 Tg C yr⁻¹). Biomass burning may account for as much as 40 per cent of the total global annual production of CO₂, estimated at 8,700 Tg C yr⁻¹ (fossil-fuel combustion producing the remaining 60 per cent) and 32 per cent of the total global annual production of CO, estimated at 1,100 Tg C yr⁻¹. The CO, non-methane hydrocarbons and NO_x produced by biomass burning lead to the photochemical production of 420 Tg of tropospheric ozone (38 per cent of the total global annual production).

Most burning of biomass is the result of human activity, and on a global scale it is increasing. R. A. Houghton (Woods Hole Research Center, Massachusetts) suggested that emissions from biomass burning may have increased by about 50 per cent since 1850. Tropospheric concentrations of CO₂, CO, CH₄, non-methane hydro-

carbons and ozone are all increasing with time; global biomass burning may make an important contribution to this increase and thus to potential global climate change.

The nitrogen cycle also can have important climatic effects. Nitrous oxide put into the atmosphere by biomass burning, is a greenhouse gas 250 times more powerful (molecule for molecule) than carbon dioxide. Nitric oxide, as well as being a photochemical precursor of ozone, a major pollutant in the troposphere, produces nitric acid, the fastest-growing component of acid rain. Hence, the new bridge in the nitrogen cycle identified by Lobert *et al.* is of more than mere technical interest. □

Joel S. Levine is in the Atmospheric Sciences Division, NASA Langley Research Center, Hampton, Virginia 23665-5225, USA.

1. Lobert, J. W., Scharffe, D. H., Hao, W. M. & Crutzen, P. J. *Nature* **346**, 552–554 (1990).
2. Soderlund, R. & Rosswall, T. *The Natural Environment and the Biogeochemical Cycles* (ed. Hutzinger, O.) 61–81 (Springer, Berlin, 1982).
3. Levine, J. S., Rogowski, R. S., Gregory, G. L., Howell, W. E. & Fishman, J. *Geophys. Res. Lett.* **8**, 357–360 (1981).
4. Crutzen, P. J., Heidt, L. E., Krasnec, J. P., Pollock, W. H. & Seiler, W. *Nature* **282**, 253–256 (1979).

Plugging the nuclear pore

Colin Dingwall

THE only known way that substances can pass between the nucleus of the cell and the surrounding cytoplasm is through the nuclear pore complex. Although studies of the detailed structure of the complex have often described centrally located plugs or granules, these structures were interpreted as macromolecules — such as ribonucleoprotein complexes — caught in transit as they moved through the pore. But in new work published in the latest issue of *Biophysical Journal*¹, Akey shows that the plug is instead an integral part of the nuclear pore complex. From an analysis of more than 4,000 nuclear pore complexes from the nuclear envelopes of *Necturus* oocytes, Akey proposes that the plug is a form of gate or transporter that can assume several configurations, and explains how these different forms relate to the transport of substrates through the pore.

The large-scale details of nuclear pore architecture are now fairly well established^{2–4}. On the cytoplasmic and nucleoplasmic faces of the pore complex, are two coaxial rings of eight subunits. These sandwich a large spoke assembly made up of eight subunits that have attachments to both rings at the edge of the complex. These subunits are much more massive than those in the rings and contribute most to the strong 8-fold symmetry character-

istic of nuclear-pore complexes (see Fig. 1). The complex has an overall diameter of 1,200 Å, a relative molecular mass of 124×10^6 (ref. 5) and is estimated to contain up to 100 different proteins¹. The central plug lies in the very centre of the complex, at the level of the radial spokes. It has a diameter of 370 Å and can be dissociated from the complex, as not every pore in a single preparation has a plug.

Akey examined nuclear envelopes that had been rapidly isolated and frozen to

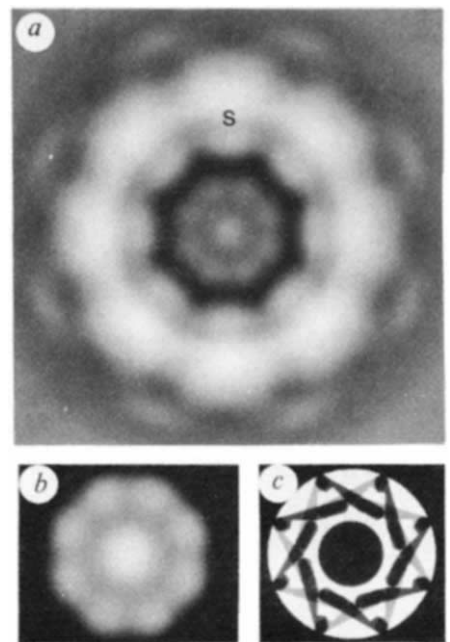


FIG. 1 *a*, Projection map of a membrane-associated nuclear pore complex showing the spokes (S) with radial arms extending beyond the ring of spokes. In the centre of the pore complex is the transporter in the in-transit state. This is shown enlarged in *b* (protein is white). *c*, Corresponding double-iris model of the transporter in the in-transit state with a substrate molecule in the centre.

liquid-nitrogen temperatures. Using techniques previously applied to the analysis of ribosomes, he sorted electron-microscope images into different groups representing different conformations of the transporter. Image analysis techniques were then used to extract structural information from these groups and to generate a projection map such as that shown in Fig. 1. In these maps, the spokes are clearly visible as are radial arms projecting beyond the edge of the complex. Most of all, the structure of the central plug (now termed the transporter) can be seen in considerable detail. The different confor-

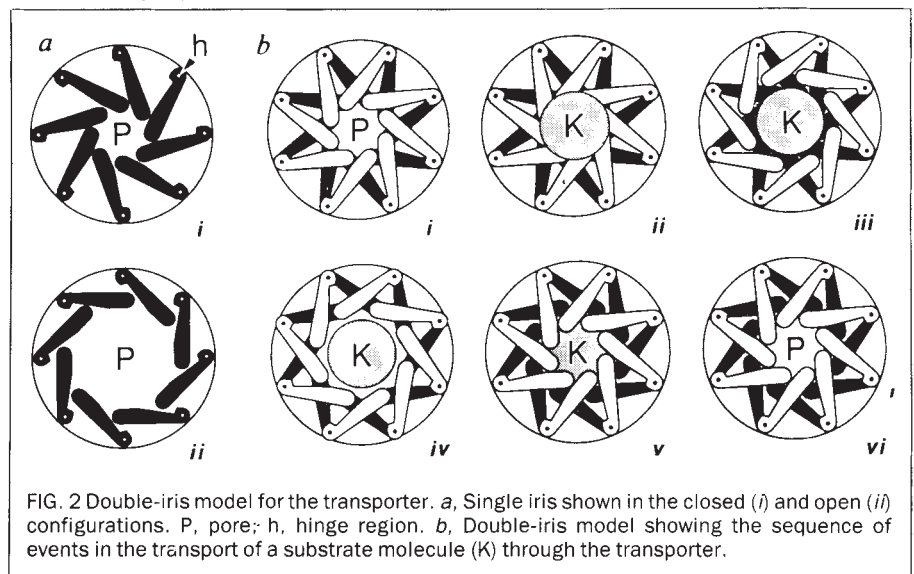


FIG. 2 Double-iris model for the transporter. *a*, Single iris shown in the closed (*i*) and open (*ii*) configurations. P, pore; h, hinge region. *b*, Double-iris model showing the sequence of events in the transport of a substrate molecule (K) through the transporter.

mational states observed are thought to represent different configurations adopted by the transporter as it transports a molecule.

This has led to the proposal of a model for the transporter in which two aligned rings of eight subunits form a double iris. In a single iris (Fig. 2*a*), the subunits can rotate about a hinge region (h) to give a closed (i) or open configuration (ii). The suggested sequence of events in the transport of a karyophilic molecule is shown in Fig. 2*b*. Both irises are initially closed (i), giving a transit pore (P) of 90 Å in diameter. A karyophilic molecule (K) docks over the central pore (ii) and the top (white) iris opens to admit the molecule (iii). Then the lower (black) iris opens (iv) and the substrate molecule moves further into the transporter creating a symmetrical 'in-transit' form. The top (white) iris closes behind the karyophilic molecule as it moves further into the expanded pore (v) before the molecule dissociates from the inner surface of the transporter (vi). The bottom (black) iris then closes in preparation for another transport cycle. The two layers of the double iris are thought to have significant contacts only at the hinge region, giving a puckered configuration when viewed in cross-section.

The model is attractive because it is consistent with several previous observations and proposals. For example, the injection of various inert molecules into cells has demonstrated the presence of a passive pore of 90 Å in diameter, which is consistent with the pore dimensions of the transporter in the closed configuration. But the pore complex can transport much larger molecules — even colloidal gold particles up to 200 Å in diameter coated with a nuclear protein⁶. Bonner⁷ suggested that the orifice of the pore could dilate in response to the presence of a transport substrate allowing entry and transport. The new model offers a credible mechanistic basis for this idea.

The pore complex can bidirectionally and apparently simultaneously transport protein into the nucleus and RNA out of it⁸. The double iris is intrinsically bidirectional and its symmetry suggests that nuclear import and export may share simi-

New livery for Caucus Racer

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

This new slimline 1990s version of the extinct dodo *Raphus cucullatus*, now on display at the Royal Museum of Scotland, Edinburgh, is the fruit of research by the Museum's Curator of Birds and Mammals, A. M. Kitchener. The dodo was discovered on the island of Mauritius in the Indian Ocean in 1592, but the depredations of mariners drove it to extinction in less than a century. A flightless member of the pigeon family, the dodo has usually been depicted as obese and faintly comical, although the classic portrait of the animal by Tenniel in Lewis Carroll's *Alice's Adventures in Wonderland* is of the wise and impartial referee of an athletic entertainment known as the Caucus Race. But this vision of corpulence stems not from observations of the birds in their natural habitat, but from paintings of captive specimens in European collections whose plump physiques may have been a symptom of confinement. The very earliest pictures, drawn in the first years of the seventeenth century, show a much sportier and more sprightly creature. Painstaking reconstruction of the few remaining skeletons, with the help of a stuffed head, a piece of beak and the cast of one foot, has allowed Kitchener and taxidermist Derek Frampton to restore the dodo more in accord with the original, realistic view of the wild bird. It now seems that the archetypal organizer of Carroll's Caucus Race may have been more fleet of foot than has been realized and the Race may have been far more competitive than the gentle jog narrated by Carroll, with the dodo as an active participant. If so, the only things lacking in the new restoration are the 'go-faster' stripes.

Henry Gee

lar properties at the level of the transporter. The pore complex contains several unusual glycoproteins (nucleoporins) with oxygen-linked *N*-acetylglucosamine residues. Lectins and antibodies to these proteins block the transport of proteins into the nucleus and bind directly to the central transporter⁹. In addition, nuclei formed *in vitro* in extracts depleted of these proteins are unable to import nuclear proteins; nuclear import is restored when glycoproteins are added¹⁰. In terms of the model, the ability of these molecules to inhibit transport would be due to cross-linking and immobilization of the transporter subunits.

An ATPase of high relative molecular

mass is enriched in preparations of the nuclear envelope and is very similar to muscle myosin heavy chain¹¹. The myosin head has similar dimensions to the subunits of the iris, so the subunits could comprise, at least in part, an ATPase. Indeed, ATP is required for transport through the nuclear pore and movement of the ATPase could generate the iris-like dilation of the transporter.

However, some aspects of nuclear-pore structure and transport are not easy to reconcile with the double-iris model. Fibres passing through the pore complex and extending into the cytoplasm and nucleoplasm¹² have been implicated as mediators of the first step in a two-step

1. Akey, C.W. *Biophys. J.* **57**, 341–375 (1990).
2. Gerace, L. & Burke, B. A. *Rev. Cell Biol.* **4**, 335–374 (1988).
3. Unwin, P.N.T. & Milligan, R.A. *J. Cell Biol.* **93**, 63–75 (1982).
4. Akey, C.W. *J. Cell Biol.* **109**, 955–970 (1989).
5. Reichelt, R. et al. *J. Cell Biol.* **110**, 883–894 (1990).
6. Feldherr, C.M., Kallenbach, E. & Schultz, X. *J. Cell Biol.* **99**, 2216–2222 (1984).
7. Bonner, W.M. in *The Cell Nucleus* Vol. 6 Part C (ed. Busch, H.) 97–148 (Academic, New York, 1978).
8. Dworetzky, S.I. & Feldherr, C.M. *J. Cell Biol.* **106**, 575–584 (1988).
9. Akey, C.W. & Goldfarb, D.S. *J. Cell Biol.* **109**, 971–982 (1989).
10. Finlay, D.R. & Forbes, D. *Cell* **60**, 17–29 (1990).
11. Berrios, M. & Fisher, P.A. *J. Cell Biol.* **103**, 711–724 (1986).
12. Maul, G.G. in *The Nuclear Envelope and the Nuclear Matrix*, 1–11 (Alan R. Liss, 1982).
13. Richardson, W.D. et al. *Cell* **52**, 655–664 (1988).
14. Borer, R.A. et al. *Cell* **56**, 379–390 (1989).

mechanism for the import of nuclear proteins¹⁵. But the relationship between these fibres and the transporter is not known. The fibres are seen in sections of whole cells, whereas Akey looked at isolated nuclear envelopes. Perhaps isolation causes disruption or detachment of the fibres.

The double-iris model, then, has its limitations. It describes only a rotational portal and cannot explain the vectorial nature of transport. Also, although transport is bidirectional, it is selective in that — broadly speaking — nuclear proteins are imported whereas RNA in the form of ribonucleoprotein particles is exported.

CELL BIOLOGY

Recipes for replication

Bruce M. Alberts

A detailed understanding of the mechanism of DNA replication requires the reconstitution of a cell-free system that reproduces the basic reaction using highly purified proteins, nucleic acids and other molecules. With such a system, each component can be individually omitted, modified and tested to ascertain its exact role in the replication process. Multi-protein replication systems of this type were first reconstituted about 15 years ago from prokaryotic sources, in particular the bacterium *Escherichia coli* and two of its viruses: bacteriophage T4 and bacteriophage T7 (refs 1,2). On page 534 of this issue, Tsurimoto, Melendy and Stillman report the establishment of a cell-free replication system from purified mammalian proteins³. By skilful manipulation of this *in vitro* system, they show that the two different DNA polymerases known to be required for eukaryotic DNA replication each have distinct roles: DNA polymerase delta makes the DNA on the leading (continuous) strand, whereas DNA polymerase alpha makes the DNA on the lagging (discontinuous) strand.

Although there is now a consensus on which proteins move a replication fork in prokaryotes (see the figure), finding the analogous components in eukaryotes has been difficult. Classical enzymology led first to the purification and characterization of polymerase alpha⁴, which copurifies with the tightly-associated eukaryotic primase⁵. Polymerase delta was obtained in a similar way⁶. But further progress — including any understanding of why two different replicative DNA polymerases should exist (there is only one in prokaryotes) — had to await the development of an extract that catalyses the replication of the genome of the monkey virus SV40 (ref. 7). Fractionation of the components of this *in vitro* replication system led to the discovery of

There are also proteins that shuttle between the nucleus and cytoplasm¹². We know little about the molecular basis of this sorting, and the transporter model lacks any feature that could explain it. Perhaps specific, soluble, receptor molecules direct substrate molecules to the transporter. Despite these qualifications, however, the new work is an important and stimulating advance in our knowledge of the structure of the nuclear pore complex. □

Colin Dingwall is in the MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK.

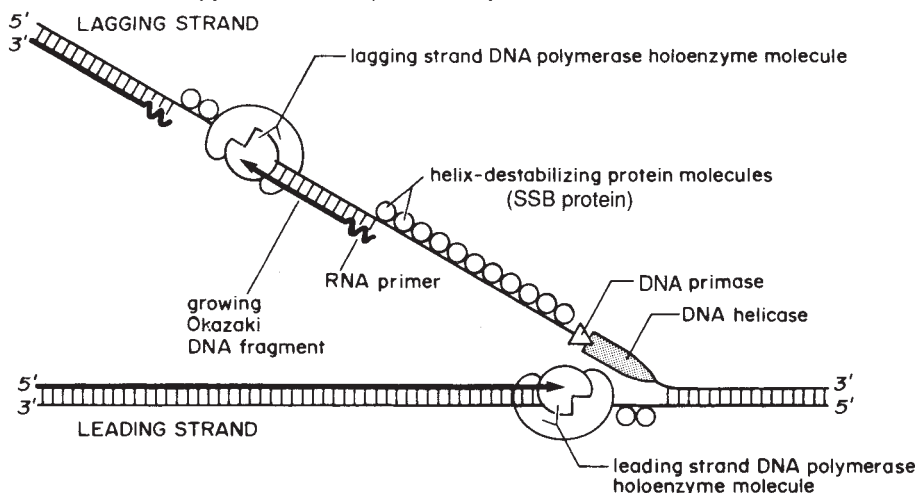
the mammalian helix-destabilizing protein (SSB; called RF-A in ref. 3) and of an important polymerase-accessory protein (called RF-C in ref. 3). Further work on this same system led to the realization that a polymerase delta accessory protein, initially discovered as a proliferating cell nuclear antigen (PCNA), is similarly an essential part of the SV40 replication system⁸.

By their development of a replication system containing only purified proteins, Tsurimoto *et al.* now suggest that the above two polymerase-accessory proteins and the SSB protein, as well as the polymerase alpha-primase complex and polymerase delta, are the main components from the host cell necessary to replicate the double-stranded circular DNA genome of the SV40 virus. A similar conclusion has recently been reached in the laboratory of T.J. Kelly (personal communication). By implication, the above proteins are thought to form the core of the apparatus that replicates

the cellular genome.

In the SV40 system (as in prokaryotes), a series of short RNA-primed DNA fragments (termed Okazaki fragments) are made by a discontinuous, 'back-stitching' process on the lagging strand, whereas much longer DNA molecules are synthesized by a continuous process on the leading strand. When polymerase delta is omitted from the *in vitro* reaction, only the lagging strand products are made. Addition of either polymerase delta or the replicative DNA polymerase from bacteriophage T4 or T7 regenerates the complete replication fork. Tsurimoto *et al.* conclude that polymerase alpha is specifically blocked from gaining access to the leading strand, and that each mammalian polymerase normally replicates one strand exclusively⁹.

The unique feature that distinguishes the SV40 replication apparatus from the corresponding cellular system is the presence of a virus-encoded protein — the SV40 T antigen. This large protein plays two essential parts in the SV40 replication process. It first binds in multiple copies to the specific 64-nucleotide DNA sequence that serves as the SV40 replication origin. Then the DNA helicase domain of the protein separates the strands of the neighbouring region of DNA helix, presumably because the complex of T-antigen molecules splits in half as it hydrolyses ATP and moves outwards in both directions from its initial site on the DNA¹⁰. Therefore, the components that are still missing from the cellular replication apparatus are an origin-binding initiator protein and a DNA helicase to move the replication fork along. In prokaryotes, these two functions reside in separate proteins: it is not known whether eukaryotes combine both functions in a single polypeptide chain (as does the SV40 virus). Finding the protein(s) that provide these two activities for mam-



DNA replication in prokaryotes. DNA polymerase on the leading strand assembles the molecule in a 5' to 3' direction. But polymerase on the lagging strand must go 'against the grain', assembling DNA from a series of RNA-primed fragments.

malian chromosome replication has become a holy grail that has thus far remained elusive.

The present SV40 *in vitro* replication system probably needs to be refined by the addition of further proteins: for example, the rate at which the replication fork moves is a tenth of that found *in vivo*, and the Okazaki fragments produced are abnormally large³. Much difficult biochemistry remains to be done, but we can expect a very sizeable payoff. Despite having a different initiator protein and DNA helicase, the SV40 virus seems likely to initiate its replication in a similar way to that of its host cell. The new results³ suggest that a very early step in this pathway is the loading of two molecules of the polymerase alpha-primase complex onto the opened-up DNA helix, with one molecule on each side of the origin of replication. If we had a blow-by-blow description of the exact pathway, we might come much closer to understanding how the transition between the

G1 and the S phases is controlled in eukaryotic cell division. In addition, we would have a very promising foundation for understanding the mysterious process whereby the cell guarantees that each region of a eukaryotic chromosome is replicated once and only once in a cell cycle. □

Bruce M. Alberts is at the Department of Biochemistry and Biophysics, School of Medicine, University of California, San Francisco, California 94143-0448, USA.

1. Alberts, B.M. *Phil. Trans. R. Soc. B* **317**, 395–420 (1987).
2. Kornberg, A. *DNA Replication* (Freeman, San Francisco, 1980).
3. Tsurimoto, T., Melendy, T. & Stillman, B. *Nature* **346**, 534–539 (1990).
4. Bollum, F.J. *J. biol. Chem.* **235**, 2399–2403 (1960).
5. Conaway, R.C. & Lehman, I.R. *Proc. natn. Acad. Sci. U.S.A.* **79**, 2523–2527 (1982).
6. Byrnes, J.J., Downey, K.M., Black, V.L. & So, A.G. *Biochemistry* **15**, 2817–2823 (1976).
7. Li, J.J. & Kelly, T.J. *Proc. natn. Acad. Sci. U.S.A.* **81**, 6973–6977 (1984).
8. Pellich, G. *et al. Nature* **326**, 517–520 (1987).
9. Borowicz, J.A., Dean, F.B., Bullock, P.A. & Hurwitz, J. *Cell* **60**, 181–184 (1990).

STELLAR EVOLUTION

Solar systems in the making

Gibor Basri

DURING the past decade, evidence has been accumulating that the curious astronomical sources known as T Tauri stars are very young solar-type stars, surrounded by great disks of gas and dust the size of the Solar System. A new article by Adams *et al.*¹ along with an earlier paper by Beckwith *et al.*² present fairly direct measurements of the masses of these disks. Most of the disks have masses above the so-called minimum solar nebula, meaning that they contain sufficient mass to produce a solar system like our own. Thus it would appear that such conditions arise quite often as part of the process of formation of a low-mass star, and also that we have the opportunity to make direct observations of the process as it occurs.

The T Tauri stars were first noticed because of the irregular variations in the intensity of their light. Then they were singled out as a class of star by the powerful emission lines in their spectra which are much brighter and broader than those seen in ordinary stars like the Sun. Their association with dark, dense interstellar molecular clouds thought to be the precursors to star formation, along with a number of other strong indicators, leaves little doubt that the stars are extremely young — just a few million years old. As observations were extended from visible light to the ultraviolet and infrared, it became clear that T Tauri stars are often much brighter in these wavelength ranges than is expected from their visible appearance.

Earlier explanations for the ultraviolet

excess included a scaled-up version of solar magnetic activity. Although it is now clear that the young stars indeed have very strong magnetic activity, seen both as powerful coronal X-ray emission and sometimes as light variations due to the presence of large dark starspots, it is also clear that the excess emission is too great to be explained solely in this way. An alternative explanation was that there is spherical cloud of material falling onto the star — the last of the collapsing interstellar cloud core. The accretion shock was invoked to explain the ultraviolet excess, and dust in the cloud re-radiating stellar light could provide the infrared excess. The main difficulty is that the amount of dust required to justify the infrared would be sufficient to block the visible and ultraviolet light that is observed.

The best features of these hypotheses are retained, and the problems eliminated, if we assume there is a magnetically active young star surrounded by a relatively flat disk of gas and dust which accretes onto the equatorial regions of the star. Stellar light re-radiated by the disk in addition to intrinsic luminosity due to gravitational heating of the disk as material spirals towards the star can neatly account for the infrared spectrum. When the material reaches the vicinity of the star it must lose its orbital kinetic energy as it accretes onto the stellar surface. This happens in a relatively small region which is therefore heated sufficiently to provide the ultraviolet excess emission. The other emission properties of T Tauri stars arise fairly

naturally from this paradigm (see refs 3 and 4 for reviews).

Disk models that fit the observations suggest that the disk is opaque where the visible and infrared light is produced. As we see only the disk's surface, it is difficult to know how much material it contains. And although the T Tauri disks are thought to be at least as big as Pluto's orbit, it is just beyond our current capability to image them directly. However, the dust becomes increasingly transparent at microwave and millimetre wavelengths, which arise in the cooler outer parts of the disk. Under these conditions the amount of emission seen is directly proportional to the total mass of emitting material. The first observations of this kind are only now becoming available^{1,2,5}. When combined with disk models they provide direct estimates of the total disk masses, which range from a few hundredths to about one solar mass. The principal uncertainty in these estimates is the exact value of the dust's opacity, which causes the mass to be uncertain by a factor of 2–5 (but probably in the direction of underestimates). The minimum solar nebula necessary for the formation of giant gas planets like Jupiter is thought to be about a hundredth of a solar mass — less than that measured for the T Tauri stars. By studying the properties of the central stars with and without circumstellar disks, Strom *et al.*⁶ have estimated that the typical disk lasts at most about 10 million years, which puts a constraint on the time for such giant planets to form.

We are now in a position to say something like one-third of newly forming stars of one solar mass or less appear to satisfy most of the conditions we believe necessary for the formation of planets. There are even a few cases in which the small dust particles near the star seem to have disappeared (by falling into the star, being blown away by its strong winds, or perhaps by agglomeration into larger particles) while the outer dust disk is still present. The final (but most important) observation still missing is of planets themselves. It is already clear from studies of older nearby solar-type stars that at best a small fraction of T Tauri disks have produced planets the mass of Jupiter or larger, and indeed the first extra-solar planet remains to be found. □

Gibor Basri is in the Astronomy Department, University of California, Berkeley, California 94720, USA.

1. Adams, F.C., Emerson, J.P. & Fuller, G.P. *Astrophys. J.* **357**, 606–620 (1990).
2. Beckwith, S.V.W., Sargent, A.J., Chini, R.S., & Güsten, R. *Astr. J.* **99**, 924–945 (1990).
3. Bertout, C. *A. Rev. Astr. Astrophys.* **27**, 351–395 (1989).
4. Mundt, R. & Appenzeller, I. *Rev. Astr. Astrophys.* **1**, 291–334 (1989).
5. Weintraub, D.A., Sandell, G. & Duncan, W.D. *Astrophys. J.* **340**, L69–L72 (1989).
6. Strom, K.E., Strom, S.E., Edwards, S., Cabrit, S. & Skrutskie, M.F. *Astr. J.* **97**, 1451–1470 (1989).

Ocean ridges in hot water

Robert J. Poreda

SCATTERED along the mid-ocean ridges — the scars that mark where plates part and new oceanic crust grows — are the hydrothermal vents where sea water sinks below the sea floor to be heated by magma and forced out again. In August 1986, Baker and colleagues¹ found that the Juan de Fuca ridge in the east Pacific had suddenly released a tremendous amount of heat — equivalent to an entire year's output from a normal ridge. The discovery of this megaplume shattered the idea that hydrothermal activity is a steady process. The outburst coincided with disruption of the normal activity in the vent field. In particular, the flux into the ocean of ³He, derived from the magma beneath the ridge, was much larger, while the heat flux remained constant. Over the following three years, as Baker and Lupton report on page 556 of this issue², that flux returned towards more normal values. The authors use this information as an indicator of the subterranean activity, and argue that the original megaplume was almost certainly triggered by an injection of magma into the ridge axis.

The measure the authors use is the ratio of the amount of ³He to temperature anomalies in the water column above a vent field. The association between the release of ³He and energy from hydrothermal vents, first noted by Jenkins *et al.* for the Galapagos vents³, provided a connection between the extraction of heat and volatiles from basaltic magma at mid-ocean ridges. Fluids from East Pacific Rise vents have a relatively restricted range of ³He/heat ratios^{4,5}.

Why should the extraction of helium and heat from cooling magmatic systems be so strictly linked when the mechanisms that control their transfer into the ocean may differ markedly? One possible reason may lie in the fact that in most hydrothermal systems the balance between helium and heat extraction is maintained through the process of fractional crystallization of the magma beneath the ridge axis. Ultimately, this is the source of both the helium and the heat. In a magmatic system which is saturated with volatiles for a particular temperature and pressure, circulating hydrothermal fluids cool the magma, causing it to crystallize. The resultant increase in volatile (especially CO₂) concentrations in the remaining melt leads to degassing. CO₂, the principal volatile species, and helium have nearly the same solubility and therefore behave similarly in basaltic melts^{7,8}.

Thus, in a fractional-crystallization model there is a one-to-one correspondence between the extraction of heat and

helium (or other volatiles) from the melt. A simple calculation demonstrates that for a typical ³He concentration in basalt⁹, $1.8 \pm 0.5 \times 10^{-10} \text{ cm}^3 \text{ g}^{-1}$, and a latent heat of fusion of 80 cal g⁻¹, 'steady-state' extraction of helium and heat from a magma would give a ³He/heat ratio of $2.2 \pm 0.5 \times 10^{-12} \text{ cm}^3 \text{ cal}^{-1}$. Adding the heat obtained from cooling the basalt from 1,000 to 300 °C reduces the ratio to $0.5 \times 10^{-12} \text{ cm}^3 \text{ cal}^{-1}$. More detailed investigations will produce an accurate estimate of the heat budget for individual spreading segments.

As shown by Baker and Lupton, only when a system such as the Juan de Fuca Ridge is perturbed does the ³He/heat ratio change dramatically. Injection of new magma rich in volatiles will directly increase the measured ³He/heat ratio. This ratio will subsequently decrease as the system returns to a steady-state condition. This is the most straightforward explanation for the origin of the megaplume. Other mechanisms proposed, such as a variable water/rock ratio, do not explain why the heat output stayed constant while ³He flux decreased. And given that the vent's ³He/heat ratio seems to be returning to a 'steady-state' level within four years, the steady-state magma body must be relatively small (as has been seen at other East Pacific Rise sites) so that the injection of new magma could create large excesses in volatile concentrations to change the ³He/heat ratio by the observed factor of five. A large magma chamber would effectively buffer the ³He/heat ratio against any new inputs of lava.

The Juan de Fuca Ridge has provided an excellent setting for the understanding of ridge crest dynamics. Perhaps the most intriguing message from Baker and Lupton's work is that accurate ³He and temperature profiles above hydrothermal vents yield detailed insights into the geology of the mid-ocean ridge. □

Robert J. Poreda is in the Department of Geological Sciences, University of Rochester, Rochester, New York 14627, USA.

1. Baker, E. T., Massoth, G. J. & Feely, R. A. *Nature* **329**, 149–151 (1987).
2. Baker, E. T. & Lupton, J. E. *Nature* **346**, 556–558 (1990).
3. Jenkins, W. J. *et al.*, *Nature* **272**, 156–158 (1978).
4. Welhan, J. A. & Craig, H. in *Hydrothermal Processes at Seafloor Spreading Centers* (eds Rona, P. *et al.*) 391–409 (Plenum, New York, 1983).
5. Merlivat, L., Pineau, F. & Javoy, M. *Earth planet. Sci. Lett.* **84**, 100–108 (1987).
6. Lupton, J. E., Baker, E. T. & Massoth, G. J. *Nature* **337**, 161–164 (1989).
7. Stolper, E. & Holloway, J. R. *Earth planet. Sci. Lett.* **87**, 397–408 (1988).
8. Jambon, A., Weber, H. & Braun, O. *Geochem. Cosmochim. Acta* **50**, 401–408 (1986).
9. Sarda, Ph. & Graham, D. *Earth planet. Sci. Lett.* **97**, 268–289 (1990).

Down with waste!

ONE ingenious suggestion for disposing of nuclear waste is simply to tip it into a geological subduction zone. As the down-going lithospheric plate sinks into the Earth, far deeper than any borehole we could make, the radioactive material will sink too, until it melts and disperses into the Earth's mantle. The geological cycle is so slow that, by the time it re-emerges at some mid-ocean ridge, it will have completely decayed, having in the process contributed to the natural radioactive heating of the planet.

Daedalus approves of this elegant scheme, and plans to make it more practical. He points out that the trenches where crustal plates are being drawn under are usually marked by chains of active volcanoes — like those of the Andes and Central America, which mark the East Pacific subduction zone. Volcanoes, of course, give quick access to a molten region of the Earth's interior. Statistically speaking, they erupt very rarely. It should be fairly safe to drill down through the solidified lava-plug of a quiescent volcano, and inject a charge of nuclear waste into the molten interior. The cold, dense waste would rapidly sink into the depths. It would dissolve and disperse in the vast subterranean molten reservoir, and with good luck would join the general downward flow of the subduction trench. Even with bad luck, an erupting volcano ejects only a tiny proportion of its total molten inventory. Very little of the injected waste would come back to us, and even that would be hygienically vitrified in the solidifying lava.

But a natural volcano is inevitably rather a worrying waste basket. So Daedalus proposes an artificial one. His plan is simply to lay a large insulating blanket over the ground of the subduction zone. At such geothermal hot spots, where molten rock is very near the surface, the Earth's outward heat flux is higher than average. The insulation will block this flow, and the temperature beneath it will rise. Sooner or later it will reach the melting point of the local rock. A molten column will then stretch from the surface right down to the subterranean lava reservoir.

Being in fair hydrostatic equilibrium, this artificial volcano should be entirely safe and quiescent. Radioactive waste, scrapped tanks and anything else of reasonable density could simply be thrown in and allowed to sink. The volcano might be maintained as a free source of energy, but big power stations are out of place in such volcanic regions. It would be better to tip all the world's nuclear waste into the volcano, and then remove the insulating blanket. The lava column would soon cool and solidify again, sealing the nasties safely in the nether regions. David Jones

Diamond and graphite precursors

SIR—Badziag *et al.*¹ have attempted to explain the observation that diamond is often a product in carbon-forming reactions. They find that, in the presence of hydrogen, precursors to diamond nucleation are of lower energy than graphite precursors because of the influence of surface C–H groups. Thus they conclude that homogeneous nucleation of diamond is preferred over graphite nucleation. But equilibrium constants, rather than energies, are the appropriate

measures of stability for nucleation precursors, because it is essential to account for entropy loss during growth. On this basis, I find that diamond precursors are always significantly less stable than graphite precursors under typical conditions, so that there are greater thermodynamic impediments to diamond nucleation than to graphite nucleation.

To compare the thermodynamic stabilities of two substances, one must consider the balanced reaction that interconverts

EQUILIBRIA BETWEEN DIAMOND AND GRAPHITE PRECURSORS

Precursor	$\Delta H_{f,298}^0$ (kcal mol ⁻¹)	$\Delta S_{f,298}^0$ (cal mol ⁻¹ K ⁻¹)	$T_c^\dagger$ (K)	$\log(K_{eq})^\ddagger$ 700 K	$\log(K_{eq})^\ddagger$ 1,000 K
Diamond					
Graphite					
cyclohexane = benzene + 3 H ₂	49.3	90.1	547	4.3	8.9
adamantane = naphthalene + 4 H ₂	67.6	130.6	517	7.3	13.8
diamantane = phenanthrene + 5 H ₂	88.5	164.9	536	8.4	16.7

Thermodynamic values from refs 4–7. Diamantane entropy is estimated from ring additivity (86.5 cal mol⁻¹K⁻¹; ref. 8).

[†] Temperature at which equilibrium constant is equal to unity (ceiling temperature).

[‡] K_{eq} is the equilibrium constant in atmospheric-pressure standard states. These equilibrium constants are equal to the relative concentrations of graphite and diamond precursors when [H₂] = 1 atm.

Child leukaemia — curies or cars?

SIR—Evans in News and Views¹ reviewed the evidence suggesting that the apparent childhood leukaemia excess near the Sellafield nuclear-fuel reprocessing plant in the United Kingdom is unlikely to be linked to preconceptional paternal radiation². Evans finds particularly telling the absence of increased risk of leukaemia in offspring of Hiroshima/Nagasaki survivors, the lack of increase of inherited congenital abnormalities near Sellafield (which would have been expected), as well as the presence of increased risk in some groups of workers outside the nuclear industry. Evans considers that occupational exposure to chemical leukemogens, such as benzene, or activation of viral leukemogens by “DNA-damaging agents” are more plausible explanations.

I would like to suggest a further, related hypothesis: that non-occupational pre- and postnatal exposure to benzene from petrol evaporation and car exhausts may be a significant contributory factor to leukaemia risk. Sudden, local expansion in car ownership and usage caused by geographically isolated industrial and/or ‘new town’ developments could result in the emergence and detection of leukaemia ‘hot spots’ as small but real statistical fluctuations above leukaemia incidence baseline.

There is considerable controversy about the leukaemia risk associated even with occupational benzene exposure. Risk estimates are derived from probably

unsatisfactory exposure estimates for cases and little is known about the shape of the benzene/leukaemia dose–response curve³. Nevertheless, permitted occupational exposure levels in the United States have recently been reduced from 10 to 1 parts per million⁴. Levels approaching this (780 parts per billion) have been recorded in cars in the United States, despite strict exhaust standards⁵. In California, ambient air levels of only 4.6 parts per billion benzene have been suggested to contribute an excess lifetime risk of 101 to 780 cases per million people exposed⁶.

Such estimates are necessarily crude as they are based on questionable ‘extrapolation-through-zero’ risk assumptions, but are derived from cases in occupationally exposed adults. By contrast, little is known about the impact of benzene (or its maternal metabolites) *in utero* or in the context of naive postnatal metabolic detoxification systems.

SIMON P. WOLFF

Department of Clinical Pharmacology,
University College London,
5 University Street,
London WC1E 6JJ, UK

- Evans, J.H. *Nature* **345**, 16–17 (1990).
- Gardner, M.J. *et al. Br. med. J.* **300**, 423–429 (1990).
- Swaen, G.M. & Meijers, J.M. *Br. J. ind. Med.* **46**, 826–830 (1990).
- Nicholson, W.J. & Landrigan, P.J. *Envir. Hlth Perspect.* **82**, 185–188 (1989).
- Gilks, J.M.L. *et al. CONCAWE: Oil Companies' European Organisation for Environmental and Health Protection*. Report no. 8/89 (1989).
- Read, R.C. & Green, M. *Br. med. J.* **300**, 761–762 (1990).

the two: the species with the higher equilibrium concentration is the more stable. Illustrative results of such calculations for three diamond precursors and the corresponding graphite precursors are shown in the table. They demonstrate that, even under relatively mild pyrolysis conditions, graphite precursors are far more stable than diamond precursors with the same number of carbon atoms.

The ‘ceiling temperatures’ in the table are temperatures above which the diamond precursors (cyclohexane, adamantane and diamantane) become less stable than the corresponding graphite precursor species in the presence of 1 atm H₂. For the three diamond precursors considered, this temperature falls between 500 K and 550 K and shows little dependence on precursor size. The favourable enthalpies of formation of the diamond precursors are overwhelmed by unfavourable entropies of reaction above this temperature. Below this temperature, equilibrium calculations show that diamond precursors are exceedingly unstable relative to methane. Reactions under these conditions would lead to dissociation, not growth.

These conclusions agree with our earlier work^{2,3} on hydrocarbon equilibria at high temperatures, which outlined the thermodynamically preferred pathways for carbon nucleation under such conditions. Growth involved polycyclic aromatic hydrocarbons (graphite precursors); the higher H/C ratios of other classes of compounds made them far less stable at high temperatures³.

In view of the extreme instability of saturated hydrocarbons at high temperatures, a mechanism for diamond nucleation that involves these species seems unlikely. A more plausible pathway involves the initial formation of highly energetic carbon species (C, C₂, CH and so on) under severe conditions, followed by quenching and coalescence to form various carbonaceous solids. Other than the energy required to form such energetic precursors, this pathway is not subject to major thermodynamic impediments.

STEPHEN E. STEIN

Chemical Kinetics Division,
National Institute of Standards and
Technology,
Gaithersburg,
Maryland 20899, USA

- Badziag, P., Verwoerd, W.S., Ellis, W.P. & Greiner, N.R. *Nature* **343**, 244–245 (1990).
- Stein, S.E. *J. phys. Chem.* **82**, 566–571 (1978).
- Stein, S.E. & Fahr, A. *J. phys. Chem.* **89**, 3714–3725 (1985).
- Stull, D.R., Westrum, E. & Sinke, G.W. *The Chemical Thermodynamics of Organic Compounds* (Wiley, New York, 1971).
- Clark, T., Knox, T., McKerver, M.A., Mackle, H. & Rooney, J.J. *J. Am. Chem. Soc.* **101**, 2404–2410 (1979).
- Boyd, R.H., Sanwal, S.N., Shary-Tehrany, S. & McNally, D. *J. phys. Chem.* **75**, 1264–1271 (1971).
- Carson, A.S. *et al. J. Chem. Thermodyn.* **3**, 915–918 (1974).
- Stein, S.E. & Barton, B.D. *Thermochim. Acta* **44**, 265–281 (1981).

Tectonic origins

SIR—Muenow *et al.*¹ provided an intriguing example of the use of volatile abundances in submarine lavas as a discriminant of tectonic origin. They demonstrated some of the problems involved in measuring primary water contents in submarine volcanic rocks and documented a distinct difference in K_2O/H_2O between ocean-island basalts and mid-ocean or back-arc basalts. But their suggestion that K_2O/H_2O values are a sufficient criterion to discriminate island-arc basalts from back-arc basin basalts is, in our view, premature.

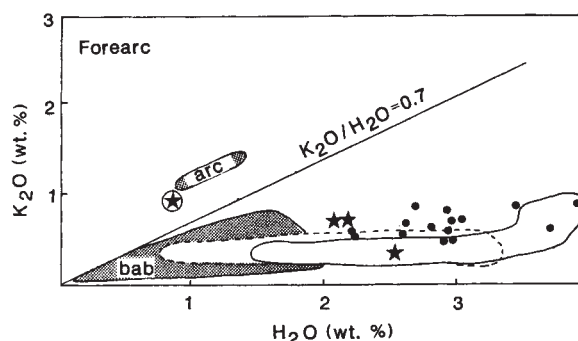
The field defined for arc volcanics by Muenow *et al.* is based on analyses of lavas from Fukujin Seamount in the northern Mariana island arc^{1,2}, which is erupting lavas of a medium- K_2O volcanic series (0.5–1.5% K_2O at 53% SiO_2). But both the ancient and active Mariana–Bonin arcs have erupted lavas with both higher and lower K_2O contents³. Active or dormant volcanos in the Mariana arc erupting low- K_2O series lavas (less than 0.5% K_2O at 53% SiO_2) include Nikko (23°05'N, 0.2–0.4% K_2O), Ruby (15°45'N, 0.45% K_2O) and Supply Reef (20°08'N, 0.4% K_2O) seamounts⁴. The oldest arc units in the Mariana–Bonin system (Eocene–Oligocene) include abundant low- K_2O series volcanics. These volcanics are tholeiites and boninites in the forearc^{5,6} and on Guam⁷, and are arc tholeiites on the Palau–Kyushu ridge⁸.

The high K_2O/H_2O of the arc field described by Muenow *et al.* is in part due to the absence of low- K_2O lavas among their analysed samples. There are few accurate analyses of volatile abundances in arc lavas, both because of the scarcity of fresh submarine arc glasses and because of the problems of separating primary from secondary water¹. The best available estimates of H_2O or total volatile concentrations in low- K_2O submarine volcanics from the Mariana–Bonin system suggest that the arc field is much broader than that defined by Muenow *et al.* (see figure). Mass spectrometric analyses of several boninitic submarine glasses and one arc tholeiitic submarine glass from forearc volcanic rocks^{5,9,10} fall at much lower K_2O/H_2O than Fukujin. Hydrogen and oxygen isotope evidence indicates that primary water contents in these rocks are 1.6 to 2.4% H_2O (ref. 5). Tholeiitic and boninitic volcanics on Guam have bulk rock

compositions similar to those of the forearc volcanics⁷; such low K_2O , low K_2O/H_2O volcanics probably comprise a large part of the initial subduction-related volcanism in the arc. Total volatile concentrations (by difference from microprobe analyses of glasses) for tholeiitic and boninitic rocks from the forearc⁶ also cover a wide range of values at low K_2O/H_2O . These are upper limits to H_2O contents. But H_2O is the most abundant volatile in arc volcanics⁷ and halving, or even quartering, most of these values will leave them in the low K_2O/H_2O field.

There are no reliable measurements of H_2O concentrations in low- K_2O volcanic glasses from the active Mariana arc. Volcanic rocks like those from Nikko or Supply reef with 0.2–0.4% K_2O would have to have less than 0.3–0.6% H_2O to have K_2O/H_2O higher than 0.7. These are much lower than values typically reported for arc volcanic rocks^{2,5,10}.

K_2O/H_2O values certainly provide a useful tool for examining the origin of submarine volcanic rocks. They may yet be a useful discriminant for arc and back-arc lavas if low- K_2O arc lavas generally have higher H_2O than back-arc basin lavas of similar K_2O content, as appears to be the case based on limited data. But without



K_2O and H_2O concentrations for low K_2O series lavas from the Mariana–Bonin forearc. Shaded fields are for arc and back-arc (bab) volcanics from ref. 1. Dots are boninite glasses with mass spectrometric H_2O values from Chichi-jima^{5,10}, stars are boninite glasses and one arc tholeiite glass from Mariana forearc with mass spectrometric H_2O values⁹. Field within solid line is for tholeiite glasses from forearc⁶ (H_2O inferred by difference from probe analyses of glasses); dotted field is the same for boninitic glasses⁶. Circled star is mass spectrometric analysis of a Fukujin Seamount glass⁹; the value is similar to those obtained by Muenow *et al.*

careful analyses of low- K_2O arc glasses, volcanic samples like those from the Troodos ophiolite¹ cannot be confidently assigned a back-arc or arc origin on the basis of K_2O/H_2O .

SHERMAN BLOOMER

Department of Geology,
Boston University,
Boston,
Massachusetts 02215, USA

ROBERT STERN

Programs in Geosciences,
University of Texas at Dallas,
Richardson,
Texas 75083–0688, USA

Toxin arrest

SIR—Bradshaw in Scientific Correspondence¹ has suggested the interesting idea that the *Kluyveromyces lactis* toxin may arrest the growth of sensitive yeast cells by interfering with cell-wall biosynthesis. The hypothesis is based on the observation that a region of the largest (α) subunit of the toxin shows sequence similarity to the chitin-binding domain of several plant chitinases.

Unfortunately, it is very unlikely that the toxin arrests sensitive cells at the G1 stage by acting as a chitinase, as Tokunaga *et al.*² have shown that the activity of the toxin resides in the γ -subunit alone. The γ -subunit has no similarity at all to chitinases³ but is insufficient alone to arrest cells when added exogenously². This suggests that the α - and β -subunits must be required for entry of toxin into the cell.

MICHAEL J. R. STARK

Department of Biochemistry,
The University,
Dundee DD1 4HN, UK

Domestic olive

SIR—Since at least the Bronze Age, the olive (*Olea europaea*) has been one of the most economically important plants in the Mediterranean basin¹. Nevertheless, little is known of the origin of the human use of this plant. The discovery of a carbonized olive pit, dated to the seventh millennium BC and associated with cultural remains, sheds light on the beginning of the association.

The olive seed is from the site of Cova de la Falguera, a rock shelter less than 10 km southwest of the town of Alcoy, midway between Valencia and Alicante. The site was discovered in 1981 and staff at the Museo Arqueológico de Alcoy made a limited test sounding². Seven stratigraphic units were defined in the excavated area, all but the lowermost containing abundant cultural material. The two lowermost artefact-bearing strata (II and III) produced assemblages of chipped stone artefacts characteristic of early Holocene hunter–gatherer societies of the Spanish Epipalaeolithic (about 9000–5000 BC).

A single carbonized seed was the only datable material recovered from stratum II, the earliest evidence for human occupation at the site. Routine identification

1. Muenow, D.W. *et al.* *Nature* **343**, 159–161 (1990).
2. Garcia, M.O. *et al.* *Geochim. cosmochim. Acta* **43**, 305–312 (1979).
3. Meijer, A. & Reagan, M. *Geology* **11**, 67–71 (1983).
4. Bloomer *et al.* *J. geophys. Res.* **94**, 4469–4496 (1989).
5. Dobson, P.F. & O'Neill, J.R. *Earth planet. sci. Lett.* **82**, 75–86 (1987).
6. Meijer, A. *et al.* *Init. Repts. DSDP* **60**, 709–730 (1981).
7. Reagan, M. & Meijer, A. *Geol. soc. Bull. Am.* **95**, 701–713 (1984).
8. Wood, D.A. *et al.* *Init. Repts. DSDP* **59**, 611–633 (1980).
9. Poreda, R.J. thesis, Univ. California (1983).
10. Dobson, P.F. thesis, Stanford Univ. (1986).

1. Bradshaw, H.D. Jr *Nature* **345**, 299 (1990).
2. Tokunaga, M., Kawamura, A. & Hishinuma, F. *Nucleic Acids Res.* **17**, 3435–3446 (1989).
3. Stark, M.J.R., Boyd, A., Mileham, A.J. & Romanos, M.A.R. *Yeast* **6**, 1–29 (1990).

of the seed before dating indicated that it was olive (*Olea spp.*) and of a size (11 mm long) generally considered domestic³. Hence, we felt that the seed was probably intrusive from the late Neolithic or early Chalcolithic strata and not associated with the Epipalaeolithic stratum II. Because the presence of olive in Bronze Age contexts has been well documented at various sites^{1,3,4}, we were not surprised by its appearance here and took no photographs of it. As part of a project to date early prehistoric sites of the region, we submitted the seed to the NSF Accelerator Facility for Isotope Analysis at the University of Arizona in the hope of obtaining a terminal radiocarbon date for the site.

Unexpectedly, the seed returned an uncalibrated date of 7410 ± 70 radiocarbon years BP, with a calibrated age of 6430–6090 BC (ref. 5). This is well within the range of the late Epipalaeolithic in eastern Spain⁶. The correspondence in age between the cultural material and the directly dated seed, and fact that the seed was carbonized, indicating it was burned, suggest that it was directly associated with the Epipalaeolithic human occupation of La Falguera. These circumstances also reduce the possibility that the seed was contaminated by older carbon.

The age of the seed, its size and its association with cultural material raises questions of whether it is a domestic olive and how the plant was used by the Epipalaeolithic inhabitants of La Falguera. Unfortunately, it is not possible to answer these questions on the basis of a single seed. Regardless of its domestic status, however, it now appears that the use of this economically important taxon began before the Neolithic 'revolution' which brought agricultural economies to western Europe.

C. MICHAEL BARTON

Department of Anthropology,
Arizona State University,
Tempe, Arizona 85287-2402, USA

FEDERICO RUBIO GOMIS

Museo Arqueológico Municipal,
03800 Alcoy, Spain

CHARLES A. MIKSICEK

Office of Arid Land Studies,
University of Arizona,
Tucson, Arizona 85721, USA

DOUGLAS J. DONAHUE

Department of Physics,
University of Arizona,
Tucson, Arizona 85721, USA

1. Zohary, D. & Hopf, M. *Domestication of Plants in the Old World* 128–136 (Clarendon, Oxford, 1988).
2. Rubio Gomis, F. & Barton, C.M. *Trabajos de Prehistoria* (in the press).
3. Renfrew, J.M. *Palaeoethnobotany* 132–133 (Columbia University Press, New York, 1973).
4. Zohary, D. & Spiegel-Roy, P. *Science* **187**, 319–327 (1975).
5. Stuiver, M. & Pearson, G.W. *Radiocarbon* **28**, 805 (1986).
6. Villaverde, V. & Martí, B. *Paleolítico / Epipaleolítico. Les Sociétés Chacadores de la Prehistoria Valenciana* 171–177 (Servei d'Investigació Prehistòrica de la Diputació de València, 1984).

Eruption terms

SIR—Our observations¹ of Etna's vigorous pyroclastic eruption in September 1989 prompt us to draw attention to a significant misapprehension concerning the kind of eruptive activity that yields an important class of volcanic deposit. 'Strombolian' is a term used by volcanologists to describe two different phenomena: a well-known style of eruptive activity and an important class of pyroclastic fall deposit. Unfortunately, the deposits commonly termed 'strombolian' are not the results of strombolian activity. We argue that basaltic scoria cones and their associated tephra deposits are not formed by strombolian activity, but by microplinian activity, similar in nature to plinian and subplinian activity, but of lesser intensity, giving rise to lower eruption columns.

In its earliest usage, the term strombolian was applied only to the style of eruption². Logically, the style of activity denoted by the term should be that displayed by Stromboli volcano itself. Most volcanologists would agree that the outstanding aspect of Stromboli's characteristic activity is that it is episodic: discrete slugs of incandescent pyroclasts are explosively ejected at intervals which may range from seconds to hours. Cotton³ followed Mercalli in defining strombolian activity as follows: "liquid lava is thrown up by explosions or gas fountains, and some of this accumulates round about as spatter, scoria and bombs. There is no dark smoke-like cloud emitted". An important consequence of the discontinuous nature of the activity is that a convecting eruption column is not sustained: material in the ejected slug rapidly falls out and only a small ash cloud is dispersed by the wind. Ejecta seldom ascend more than 200 to 300 metres above the vent.

Deposits that have been termed strombolian include basaltic scoria cones and air-fall mantles of tephra that have measurable thicknesses several kilometres from the vent. Thus, detailed studies of the distribution of grain sizes are possible, and the deposits have been well characterized in terms of the fragmentation and dispersal indices^{4,5}. Formation of such deposits has often been observed, for example during the 1973 eruption of Heimaey, Iceland⁶, and the 1975–76 Great Tolbachik Fissure eruption, Kamchatka⁷, so the nature of the activity giving rise to them is well documented. Rather than discrete slugs of material being ejected as in strombolian activity *sensu stricto*, sustained blasts take place, which may continue for periods of several hours. During the Great Tolbachik Fissure eruption, for example, there was "steady, non-stop emission of an enormous volume of cinders, bombs and ash in a mighty gas jet. The incandescent pyroclastic material

formed a fiery torch up to 1.2 km high"⁷. Sustained convection carried the eruption column at Tolbachik to a height of 10 km, almost two orders of magnitude higher than strombolian bursts *sensu stricto*. Similar behaviour has been observed in many other basaltic pyroclastic eruptions, including that at Etna in 1989 where there was commonly a sustained tephra column 300–400 m high¹.

These studies demonstrate that 'strombolian' tephra deposits form an important and easily defined volcanic phenomenon. They do not, however, result from true strombolian eruptive activity. It has been clear for some time that there is no basic difference in eruption mechanism between the largest basaltic pyroclastic eruptions producing widespread plinian basaltic scoria deposits (such as Tarawera 1886) and those of silicic eruptions producing plinian pumice deposits⁸. Similarly, differences between eruptions producing widely dispersed basaltic plinian deposits and those producing less dispersed basaltic 'strombolian' deposits are only differences in magnitude (the total erupted mass) and intensity (the magma discharge rate). The deposits form a continuous size spectrum⁹.

We suggest using the term microplinian for the basaltic scoria fall deposits with grain size and distribution characteristics currently described as strombolian, and for the style of eruptive activity giving rise to them. This would emphasize the continuity of the spectrum of pyroclastic fall deposits, and would be consistent with other terms such as subplinian, ultra-plinian and phreatoplinian (see ref. 10). It would also depict more accurately the nature of the eruption process which gives rise to the deposits.

P. W. FRANCIS

Department of Planetary Geosciences,
University of Hawaii, Honolulu,
Hawaii 96822, USA

L. S. GLAZE

D. PIERI

Jet Propulsion Laboratory,
Pasadena, California 91109, USA

C. M. M. OPPENHEIMER

D. A. ROTHERY

Department of Earth Sciences,
Open University,
Milton Keynes MK7 6AA, UK

1. SEAN Bulletin **14**, No. 9 (2–5 September 1989).
2. Mercalli, G. *Natura delle eruzioni dell. Stromboli Att. Istit. Sci. Nat.* **24**, 105–135 (1988).
3. Cotton, C.A. *Volcanoes as Landscape Forms* (Whitcombe and Tombs, Christchurch, New Zealand, 1944).
4. Walker, G.P.L. *Geol. Rundsch.* **62**, 431–446 (1973).
5. Pyle, D.M. *Bull. Volcan.* **51**, 1–15 (1989).
6. Self, S. Sparks, R.S.J., Booth, B. & Walker, G.P.L. *Geol. Mag.* **111**, 539–548 (1974).
7. Budnikov, V.A., Markhinin, Ye. K. & Ovsyannikov, A.A. in *The Great Tolbachik Fissure Eruption: Geological and Geophysical Data 1975–1976* (eds Fedotov, S.A. Markhinin, Ye. K. 41–56 English translation (Cambridge University Press, 1983).
8. Walker, G.P.L., Self, S. & Wilson, L.J. *volcan. Geotherm. Res.* **21**, 61–78 (1984).
9. Carey, S. & Sigurdsson, H. *Bull. Volcan.* **51**, 28 (1989).
10. Cas, R.A.F. & Wright, J.V. *Volcanic Successions, Modern and Ancient* (Allen and Unwin, London, 1987).

Flying lemurs and other animals

Pride and prejudice

Don C. Des Jarlais

Covering the Plague. Aids and the American Media. By James Kinsella. *Rutgers University Press*: 1990. Pp. 229. \$22.95.

Aids. A Moral Issue. The Ethical, Legal and Social Aspects. Edited by Brenda Almond. *Macmillan*: 1990. Pp. 186. Hbk £35; pbk £14.99.

Aids and the Courts. Edited by Clark C. Abt and Kathleen M. Hardy. *ABT Books Inc*: 1990. Pp. 392. Hbk \$40; pbk \$30.

THE sociology of the AIDS epidemic is at least as complex as the virology. AIDS could literally decimate some cities in the developing world, has led to massive behavioural changes among homosexual men and intravenous drug users, is rewriting the rules for the testing of drugs for treating fatal diseases, and has led to civil disobedience by AIDS activists and by parents of children whose classmates have AIDS. Financial support for research into these social and behavioural issues has been relatively scarce, and much of our knowledge about them comes from journalistic reports and legal or ethical examinations of conflicts that have developed as the epidemic has become established.

Covering the Plague is a journalist's account of US media coverage of the AIDS epidemic. Kinsella attempts to document not just the "who, where, when" of media coverage, but also the "how and why". He addresses important questions such as the relative lack of coverage early on (attributed to prejudice against homosexuals, a general lack of scientific competence in the media, and the common practice among journalists to "follow the trend-setters"). He describes the heroic efforts of individual journalists to get the epidemic into the media (attributed to personal involvement and an ability to "show the human interest story behind the science"), and the turning point in the quantity of media coverage in the United States (when it was discovered that the actor Rock Hudson had developed AIDS).

Kinsella provides a very readable, generally excellent account of US media coverage of AIDS. There is sufficient sociological and psychological discussion that social scientists may be tempted to test quantitatively some of Kinsella's arguments. There are only two areas in which the book can be faulted as analytical journalism. One is that Kinsella looks at media coverage of AIDS among white homosexual men and among children, not of all Americans with AIDS. In the last chapters, Kinsella acknowledges that ethnic minority groups and intravenous drug users have been greatly over-represented among people with AIDS and greatly under-represented in the media coverage, but his analyses of these issues are cursory. He does not mention, for example,

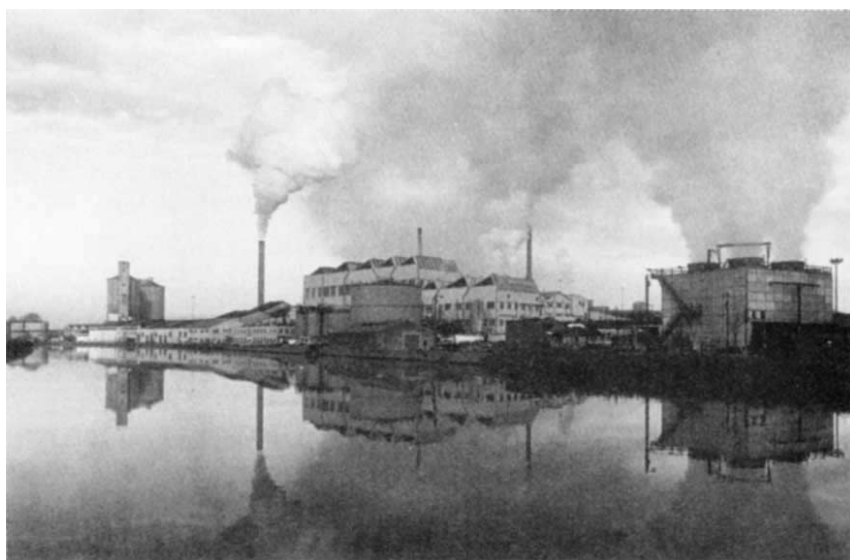
the 'hot' media topic of syringe exchanges. The second problem is the insufficient analysis of why and how researchers, public-health officials and AIDS activists have attempted to manipulate the media for public-health (and other) reasons. In the early days, these groups were not simply disinterested providers of information, and those who survived became sophisticated in their dealings with the media.

Much public debate about AIDS prevention has been framed as a conflict between the civil rights of people likely to be infected with HIV and the 'public health' rights of those who are not infected. *AIDS: A Moral Issue*, edited by Brenda Almond, examines the philosophical bases of such conflicts. The book is the proceedings of a conference held in 1986. The meeting was not planned as a 'consensus' conference, but broad agreement emerged concerning mutual rights and obligations. The community as a whole has the obligation of providing health care for people infected by HIV. People with or at risk of infection have the obligation to not transmit the virus, which

includes learning their own HIV status and informing sexual (and needle-sharing) partners of potential risks. These mutual rights and obligations are based on infected and uninfected people belonging to the same "community of trust, mutual concern and non-maleficence" (G. Gillett, page 59). Almost all the chief ethical concerns about AIDS are addressed here, and the essays demonstrate how apparent ethical problems can be resolved within the framework of a caring "moral community".

What is raised but not resolved is how to act ethically if the community is not caring and moral. The examples of discrimination against people with AIDS in all the books under review here clearly demonstrate that such a community does not exist at present. Some people believe that AIDS is the punishment of God for immoral behaviour, laws have been passed outlawing many types of sexual or drug-use behaviour, there are cases of violence against homosexuals, failures of government and other agencies to fund AIDS research, and few attempts to educate people to prevent risky behaviour.

Part of what happens in the absence of a consensual moral community is that the law becomes involved. *AIDS and the Courts*, edited by Abt and Hardy, is the proceedings of a conference for lawyers and judges. With 50 contributors, the book comes close to being comprehensive; the scholarship ranges from the well referenced article to edited transcripts. Together with *AIDS and the Law: A Guide for the Public* (Yale University Press, 1987), this will be a critical resource



British Sugar Corporation at Cantley, on the River Yare. The wetlands of East Anglia — from the Norfolk Broads to the fens and coastal marshes — are the subject of *Wetlands: An Exploration of the Lost Wilderness of East Anglia*, by David Bellamy and Brendan Quayle. The authors examine the landscape of the wetlands; the people who make a living from age-old practices; the traditions and myths that surround the area and the future of the wetlands. Many of the problems afflicting the Broads themselves are products of their popularity — the authors call for more 'green' tourism and a greater appreciation of the wetlands that still remain. Published by Sidgwick and Jackson, £15.95. □

AIDS TARGETED INFORMATION/ATIN

TARGETS YOUR LITERATURE SEARCH

Expand your base of knowledge about AIDS/HIV with *AIDS Targeted Information/ATIN*. Every month, *ATIN* not only provides abstracts but also in-depth evaluations of the current published scientific literature on AIDS. Written by clinicians and researchers for clinicians and researchers, *ATIN* provides an authoritative command of the world's literature on AIDS. Published by Williams & Wilkins. Indexed. 12 issues per year. \$125.

To order call
1-800-638-6423

Sponsored by the American Foundation
for AIDS Research (AmFAR).

Reader Service No. 430

Guarding the Guardians: Research on Editorial Peer Review

The American Medical Association announces the Second International Congress on Peer Review in Biomedical Publication to be held in September 1992. We solicit original research on the following:

- Peer review and editorial decision making in different journals
- Relationships between authors, editors, and reviewers, and how each is selected and evaluated
- Allocation of responsibility for published material
- Quality assurance, breakdowns, weaknesses, and biases

For more information contact:
Jane Smith, *BMJ*, BMA House,
London WC1H 9JR; 01-387-4499.

**Plan your
research now!**



for people dealing with AIDS within the legal system and for legislators. The case studies of discrimination experienced by people with AIDS are particularly poignant.

The overall conclusion on reading the Abt and Hardy book is similar to that drawn by several contributors to the Almond volume: the legal and judicial systems are a cumbersome method for resolving AIDS-related disputes and particularly ineffective in preventing the risk of HIV transmission. As Gostin notes, much legislation has been concerned with mandatory testing in the false belief that such testing will "do something" about the epidemic by protecting "us" from "them" who have the virus and are threatening to spread it.

Given the limitations of the mass media and of laws for creating a consensual moral community, how might AIDS be dealt with ethically and effectively? A partial solution would be to use behavioural research in making decisions. Value conflicts are often entwined with arguments about how people will act in certain circumstances — such as what will lead people to seek HIV testing, or what will "encourage" certain types of drug use or sexual behaviour. Although such conflicts cannot be resolved by data, the behavioural sciences should at least be able to refute stereotypes of sexual and drug-use behaviour. But even with the best of biomedical and social-science research at the disposal of the community, AIDS is likely to continue to highlight social problems throughout the world. □

Don C. Des Jarlais is at Beth Israel Medical Center, 1st Avenue and 16th St, New York, New York 10003, USA.

Other new AIDS books

■ *AIDS: Sexual Behavior and Intravenous Drug Use* by the National Research Council, published by the National Academy Press, is available in hardback at \$34.95 and paperback at \$24.95. In this book, members of the US Committee on AIDS Research ask how people can be encouraged to stop "risky" behaviour by examining data on the prevalence of HIV infection, patterns of sexual behaviour, the relationship between AIDS and intravenous drug use and social barriers to AIDS prevention. Also published by the National Academy Press is *AIDS: The Second Decade*, edited by H. G. Miller, C. S. Turner and L. E. Moses. Price is hbk \$34.95, £30; pbk \$24.95, £21.45.

■ Edited by Paul Volberding and Mark Jacobson is *AIDS Clinical Review 1990*, published by Dekker at \$89.75. This book is the second in a proposed series of yearly updates for clinicians in which issues related to AIDS are discussed in depth.

■ Recently published by Cold Spring Harbor Laboratory Press is *Vaccines 90*, subtitled *Modern Approaches to New Vaccines Including Prevention of AIDS*. Edited by F. Brown, R. M. Chanock, H. S. Ginsberg and R. Lerner, molecular biologists, microbiologists and clinical investigators present their latest results. Price is \$100 (paperback). □

Guided tour

Robert Cedergren

Genetic Maps. Locus Maps of Complex Genomes*, 5th edn. Edited by Stephen J. O'Brien. Cold Spring Harbor Laboratory: 1990. Pp.1,104. Hbk \$150; pbk in six volumes \$27 each.

TO CREATE a compendium where every gene can take its rightful place is the noble and largely realized goal established by Stephen J. O'Brien in *Genetic Maps*. Relying on contributors whose expertise span the complexity and evolutionary gap of the mitochondrial genome of *Aspergillus nidulans* to the nuclear genome of *Zea mays*, more than 100 genomes in all, O'Brien provides an essential resource for geneticists, molecular and cellular biologists, evolutionists and mega-sequencers alike. This compendium testifies to the explosion of information that has taken place in the identification of genetic loci in organisms, representing an extremely diverse cross-section of known phyla.

Despite the size and breadth of this remarkable volume, *Genetic Maps*, not unlike the genomes represented therein, suffers from the occasional deletion. Particularly notable by their absence are some of the mitochondrial genomes that have been fully and partially sequenced. In addition, archaeobacteria have gone unnoticed, presumably because there are no extensive gene identification and localization data — although other organisms represented by very sparse data sets are included.

Given the gargantuan task of compiling and coordinating this volume, it is indeed a shame that the result is not entirely satisfactory. There is an unequal style of presentation: some maps are fully documented but others not at all, and there is an occasional use of complex and confusing illustrations. Few illustrations attain the high quality of the T4 bacteriophage map presented by Kutter *et al.* which summarizes an immense amount of information in a pleasing and accessible form. To be sure, the effort of producing this compendium would have been even greater had stricter criteria been imposed on the type of information and the quality of illustrations, but this would have substantially increased the usefulness of the end result. In many cases, contributors could have made their information more accessible without adding substantially to their burden, but considerably lightening the load on the editor and the reader.

To take up O'Brien's metaphor that compiling this *magnum opus* "was equiv-

*Book 1, *Viruses*; book 2, *Bacteria, Protozoa, and Algae*; book 3, *Lower Eukaryotes*; book 4, *Nonhuman Vertebrates*; book 5, *Human Maps*; book 6, *Plants*.

alent to proof-reading a telephone book", the human genome data, which occupy a quarter of the volume, are far and away the "Jones" of this directory. For comparison, this space is roughly that required to print about one thousandth of the entire genome sequence in the unreadably minute fonts used in some illustrations — and gene maps have a much higher information density. This is not to say that the information presented about human genes is in its most lucid form. For example, rather than presentation of complete composite genetic and physical human maps, this information is scattered in at least five different contributions. The chapter by White *et al.* takes 'cut and paste' editing to new heights: it contains eleven "Fig. 1s", obviously extracted from different publications by this group. The inclusion of appropriate legends with these figures, however, does reduce the confusion generated by this recombinatory event. On the other hand, the highlight of the human gene section, and perhaps the entire compendium, is the simple, one-page illustration comparing mouse and human gene maps attributed to Edwards and used in the chapter by McKusik. Similar comparisons between gene maps of other organisms would have been a welcome complement to the present volume. The *Escherichia coli* map reproduced in this edition is not the latest version, surely surprising for the best characterized genome of all organisms.

After surveying this book, I cannot help wondering if the time has come to consider how to generalize more completely this sort of information, especially in the light of the expected increase in gene mapping data and its potential value. Establishing a common format for contributions, data selection and illustrations would go a long way towards this end. Continual confrontation with new symbologies and data varieties leaves one hard put to accept the assertion that a picture is worth 1,000 words. A case in point is the final drawing by Josephs and Wong-Staal in the otherwise highly informative chapter on HIV maps. Although this illustration must be related to the preceding restriction maps, it looks more like the bar codes used for pricing in supermarkets. This impression could have been avoided had a legend been included.

In addition, some sort of consensus must develop around the symbols and nomenclature of genes. Too often, but for understandable reasons, nomenclature is parochial. How much more valuable these data would be if it were possible to trace the fate of one, several or all genes through a number of organisms. Who knows what new information concerning the evolutionary significance of gene rearrangements could be inferred? Unfortunately, these problems cannot be addressed when the data are presented in this form.

It would not be fair to the editor, to his collaborators nor to the generally excellent compendium they have created not to mention a few of the outstanding chapters, since many contributions are clear, crisp and indicative of considerable dedication. Standing out in this regard are the chapters on the genomes of *Saccharomyces cerevisiae*, *Salmonella typhimurium*, *Bacillus subtilis* and *Zea mays*. In the last two, I was frankly surprised by the number of known markers.

All in all, the considerable value of this resource makes the book highly recommendable. But I expect its publication may provide the impetus for people to look for new ways to increase the accessibility of these data. □

Robert Cedergren, a fellow of the Canadian Institute for Advanced Research, is in the Département de Biochimie, Université de Montréal, Montréal, Quebec H3C 3J7, Canada.

Rational faith

John Hedley Brooke

The Correspondence of Charles Darwin. Vol. 5. 1851–1855. Edited by Frederick Burkhardt and the late Sydney Smith. Cambridge University Press: 1990. Pp. 705. £32.50, \$39.50.

TO DESCRIBE Britain's greatest naturalist as a hermit who, by manipulating both nature and people, mastered all the world

volume shows him manipulating nature as dissector of the cirripedes, as a hands-on student of hybridization and as author of ingenious experiments to test his hypothesis that flora and fauna had been distributed by the accidental transportation of seeds and ova, rather than via the conjectural land-bridges favoured by contemporaries.

We find him in the spring of 1855 soaking seeds in salt water for controlled periods to see whether they might be capable of generation after transport by swift ocean currents. And we find

him manipulating people in that, with disarming candour, he would impose on correspondents to perform experiments and to root out information on his behalf. He proudly told Joseph Hooker in April 1855 that John Davy "has been experimenting at my request (in order to see how fishes' ova might get transported) on the retention of vitality".

His astonishing thoroughness in mastering all the world is evident in his laborious work on the barnacles ("praise be to Heaven", he wrote in his first letter of 1851. "I have finished Balanus"), over which he felt he had become a bore but which, in reality, persuaded him of a far greater intra-specific variation than he had ever anticipated. As he told one correspondent in June 1851, his goal was nothing less than a monograph on "the Anatomy and Classification

Charles Robert Darwin — modesty of declared intentions.

might be considered tendentious and unflattering. Yet such a picture would not violate the images that spring from the latest volume of Darwin's edited correspondence.

Darwin himself writes that his life at Down is that of a hermit, debilitated though comfortable enough with his "three hours of daily work". Scrupulously edited and annotated as before, this new

of all the Cirripedes in the whole world".

Volume 5 of this series will probably appeal more to the Darwin connoisseur than to the general student of darwiniana. This period of his life lacks the excitement and the strong primary colours of the Beagle voyage; it lacks the supremely creative sparks that were flying in the autumn of 1838; nor, until the September of 1854, does he tell Hooker that, having

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

sent 10,000 barnacles out of the house, is he at last returning to his "old notes on species". That crucial theoretical insight, through which he finally understood why natural selection would favour divergence, belongs to the sequel.

Yet to suggest that these were uneventful years would be insensitive to a personal tragedy and a professional success (Darwin's first public recognition came with a Royal Society medal in 1853) that were of enduring significance. One of many exemplary features of this scholarly project is that each volume is a self-contained book with its own index and appendices.

Volume 5 is framed by the tragic death of Darwin's dearest child. To read the letters between Charles and Emma as hopes for Annie's recovery were cherished, revived and chilled is a poignant experience. According to a recent study by James Moore, it was this unrationalizable loss that finally drained Darwin of

any residual belief in christianity. And a world in which suffering could not be rationalized was a world not inhospitable to the exigencies of natural selection. As one takes leave of Darwin in 1855, however, one cannot fail to be struck by the modesty of his declared intentions. Repeatedly he writes that all he is about to show is that, with reference to the mutability of species, there are arguments on both sides. The concluding chapter of his *Origin of Species* would not display such modesty. For further insight into his change of emphasis, we must wait for volume 6, but it may be suggestive that, in March 1855, he was also confessing to Hooker that he found it "disgusting and humiliating to see directly opposite conclusions drawn from the same facts". □

John Hedley Brooke is in the Department of History, University of Lancaster, Lancaster LA1 4YG, UK.

A calculated endeavour

Jon V. Pepper

The History of Modern Mathematics. Vol. I: Ideas and Their Reception. Vol. II: Institutions and Applications. Edited by David E. Rowe and John McCleary. *Academic*: 1989. Pp.453, 325. Vol. I £28.50, \$39.50; Vol. II £27, \$37.50.

DESPITE the title and two substantial volumes, readers will not find here a comprehensive account of the development of modern mathematics, nor can symposium proceedings on specific and largely technical topics, such as these, be expected to provide a uniform coverage of the topics included. Nevertheless, it is commendable and useful to have this work made available so soon after the actual meeting in 1988; one of the articles is in French (with an English abstract), the remainder in English.

While the editors define the modern era in mathematics to be the period from 1801 (when Gauss's *Disquisitiones Arithmeticae* appeared) to the advent of modern computing in about 1950, the preponderance of contributions deal with work in the mid to late nineteenth century, and only 3 of the 24 chapters exclusively with the earlier twentieth century. The terminology implies the question of how to describe the past 40 years — "post-modern", perhaps?

In the later eighteenth and early nineteenth centuries the centre of mathematical development was in Paris, and Gauss himself virtually the only mathematician of note working in Germany. As the

century moved on, German work, and in particular that at Göttingen, became more dominant. This was not entirely due to the presence of Gauss, who was notoriously uninterested in teaching, nor to the later contributions of Riemann, also at Göttingen, whose personal influence would probably have been slight, even had he not died early. Klein in his youth was regarded as a giant, but after his breakdown his greatest influence for the next 40 years lay in education and organization, concerning which he was tireless both at home and abroad, particularly in the United States. The developing US university departments were staffed largely by those educated in Germany, initially by those Germans seeking a career outside the crowded domestic scene, and later by Americans themselves.

The contributions of this symposium reflect this context. The implicit development of group theory had started in France with Lagrange in 1770 and the later work of Cauchy and Galois, much of it not published until the 1840s. Galois's work was closely studied by Dedekind who provided his students at Göttingen with new results in the late 1850s. Later, Camille Jordan began to classify euclidean three-space isometries and published his *Traité des Substitutions* in 1870, a textbook with a lasting effect. Erhard Scholz, in his contribution to this volume, relates this to the earlier work of Bravais on crystal structure and the group-theoretic work at about the same time of Sophus Lie and Klein, who had first met in Berlin in 1869. Another contributor, David Rowe, discusses this work in its relation to line geometry, which Lie and Klein had learned directly or indirectly from Plücker's work at Bonn. Much of this early work of Klein and Lie was little noticed at the

time, but had later effects on Lie's theory of substitution groups and the subsequent fame of Klein's so-called Erlangen programme. Jeremy Gray, discussing algebraic geometry in the late nineteenth century, also touches on this, but his main task is to follow the subject along the path taken by the Italians, Cremona and Segre, who followed those such as Riemann, Clebsch and Max Noether, bringing analysis to the theory of curves. Gray shows, incidentally, that it is possible to discuss such matters free from a sea of symbols, but this does not mean that his discussion is less profound.

Roger Cooke contributes a chapter on Abel's earlier work which lies at the basis of these developments, and Karen Parshall one on nineteenth century invariant theory. The eighteenth century origins of duality in spherical trigonometry are well covered by Karine Chemla.

One important theme considered here is that of the relationship of pure to applied mathematics, and the means to promote public awareness of their use and importance. As the nineteenth century progressed, the universalism of Gauss dissolved in the particularism of individual mathematicians. In mathematics departments, applied mathematics was no longer important, and few such appointments made, while the outside world saw little of use in what remained. Gert Schubring and Renate Tobies in separate chapters both write perceptively on Klein's attempts to face this problem and re-orient the curriculum accordingly. That he was not very successful in this comes out in Larry Owens' chapter discussing the attempts of mathematicians in the United States in the early 1940s to convince government agencies that they had something to offer the war effort. Although some saw that the influence of the subject (and its practitioners) would come to depend on this, they found it harder to be influential with committees dominated by physicists, engineers and chemists, and their own personal jealousies sometimes did not help. The autonomy of the subject and the existence of a profession are long-lasting problems.

Three chapters deal with work on the foundations of mathematics by Cantor and Kronecker, where the continuum problem, for example, continues as a basis for current work. Although these volumes do not give the overview implied by their title, the individual contributions will be valuable to those working in their specific fields, and some have a more general interest, not least in contributing to the continuing debate about the nature of the history of mathematics and of mathematics policy. □

Jon V. Pepper is in the Department of Mathematics, University College London, Gower Street, London WC1E 6BT, UK.

Venus geology and tectonics: hotspot and crustal spreading models and questions for the Magellan mission

James W. Head & L. S. Crumpler

Spacecraft and ground-based observations of Venus have revealed a geologically young and active surface—with volcanoes, rift zones, orogenic belts and evidence for hotspots and crustal spreading—yet the processes responsible for these features cannot be identified from the available data. The Magellan spacecraft will acquire an unprecedented global data set which will provide a comprehensive and well resolved view of the planet. This will permit global geological mapping, an assessment of the style and relative importance of geological processes, and will help in the understanding of links between the surface geology and mantle dynamics of this Earth-like planet.

EXPLORATION of the planets has revealed several different mechanisms for the transfer of heat across the outer thermal boundary layer (the lithosphere)^{1,2} and several mechanisms for formation and evolution of the crust^{3,4}. These mechanisms are characterized by distinctive styles and patterns of tectonism and volcanism. The smaller terrestrial planetary bodies (the Moon, Mercury and Mars) apparently formed initial primary crusts³ through accretional heating and then lost heat over most of their history primarily by conduction. A further stage of crustal development involved secondary crust formation³, in which partial melting of the mantle produced deposits like the lunar mare basalts and a small amount of heat was lost advectively. There have been low levels of tectonic and volcanic activity on these small bodies, their lithospheres have been stable and tectonic movement has been predominantly vertical. In contrast, the Galilean satellite of Jupiter, Io, loses heat primarily by advection at multiple eruptive centres, and is characterized by many large volcanoes, subtle tectonic features, and very rapid rates of global resurfacing. On Earth, ~62% of the heat derived from the mantle is transferred across the surface thermal boundary layer by crustal spreading (a type of secondary crust formation³) and lithospheric plate recycling, in which the oceanic crust and lithosphere are created at rise crests, cool and subside as they spread laterally, and are subducted back into the mantle at convergent plate boundaries. Volcanism and tectonism are linked primarily to boundaries of the segmented and laterally moving lithospheric plates. Tertiary crust³, which forms by the reprocessing of secondary crusts, characterizes much of the continental regions of the Earth.

A fundamental question in planetary science has been the nature of the volcanic and tectonic activity, and the mechanisms of lithospheric heat transport and crustal formation and evolution, on Venus, a planet similar to the Earth in size, density and position in the Solar System. The earliest data were not of sufficient resolution to identify with confidence and to characterize the tectonic and volcanic features that would permit assessment of the modes of crustal formation³ and lithospheric heat transport². Knowledge of the high surface temperature of Venus and predictions that the basalt-eclogite transition would be deeper than on Earth, led to predictions that its lithosphere would be positively buoyant, that subduction would be inhibited, and that crustal spreading and plate tectonics would not occur on Venus⁵. Global topography data acquired by the Pioneer-Venus mission^{6,7} (Fig. 1) permitted preliminary assessments of tectonic and volcanic styles, and it was proposed that most of the topography and heat loss could be accounted for by regional hotspots⁸, that there was no evidence of subduction zones⁹, and that ≤15% of the present heat loss on Venus could

be accounted for by plate recycling¹⁰. Pioneer-Venus equatorial gravity data revealed a strong positive correlation of gravity and topography at long wavelengths¹¹, in contrast to the Earth where long-wavelength gravity anomalies are not correlated so well with topography and are thought to be due to deeper mantle convection. Gravity anomalies of large magnitude were noted in the equatorial region, and on the basis of the calculated apparent depths of compensation it was concluded that dynamic support mechanisms such as plumes or hotspots were required¹². A review of the various hypotheses at this time² concluded that the data were insufficient to rule out any of the major heat-loss mechanisms (conduction, hotspots, crustal spreading and plate recycling), that a combination of mechanisms may operate, and that the surface topography and units of Venus are young relative to the surfaces of the smaller terrestrial planets. The nature of processes of crustal formation and evolution were not explicitly discussed.

Since then, the acquisition and analysis of higher-resolution imaging and altimetry data (from Earth-based observatories^{13,14} and the Venera 15 and 16 missions¹⁵) have allowed more detailed characterization of several regions such as Beta Regio, Aphrodite Terra and Ishtar Terra (Fig. 1). Here we combine these results to develop an understanding of volcanism, tectonics, mechanisms of global heat transfer, and processes of crustal formation and evolution. For each area, we outline what is presently known and give several interpretations of the observations. We conclude with some outstanding questions that will be addressed by the global high-resolution imagery and improved altimetry and gravity data acquired by Magellan¹⁶.

Regional volcanism and tectonism

The topography of Venus is unimodal in terms of its altitude-frequency distribution^{7,17} but exhibits distinct high and low areas (Fig. 1). High topography can be produced by a variety of mechanisms (such as tectonic crustal thickening, volcanic construction and thermal uplift), each of which has distinctive geological and tectonic signatures². In this section we analyse the nature of several upland plateaus and highlands to assess their modes of origin.

Beta Regio. This 2,000 × 2,300 km equatorial highland¹⁸ rises >5 km above the mean planetary radius and contains a central N-S linear trough (Devana Chasma) 200–300 km wide (Figs 1, 2a). Correlation of Arecibo Observatory (Puerto Rico) and Venera 15–16 data¹⁹ show that there is a concentration of faults in Devana Chasma, and the Arecibo data indicate that Theia and Rhea Montes are shield volcanoes situated in and adjacent to Devana Chasma²⁰. A large positive gravity anomaly is centred on the topographic rise; simple isostatic models suggest an

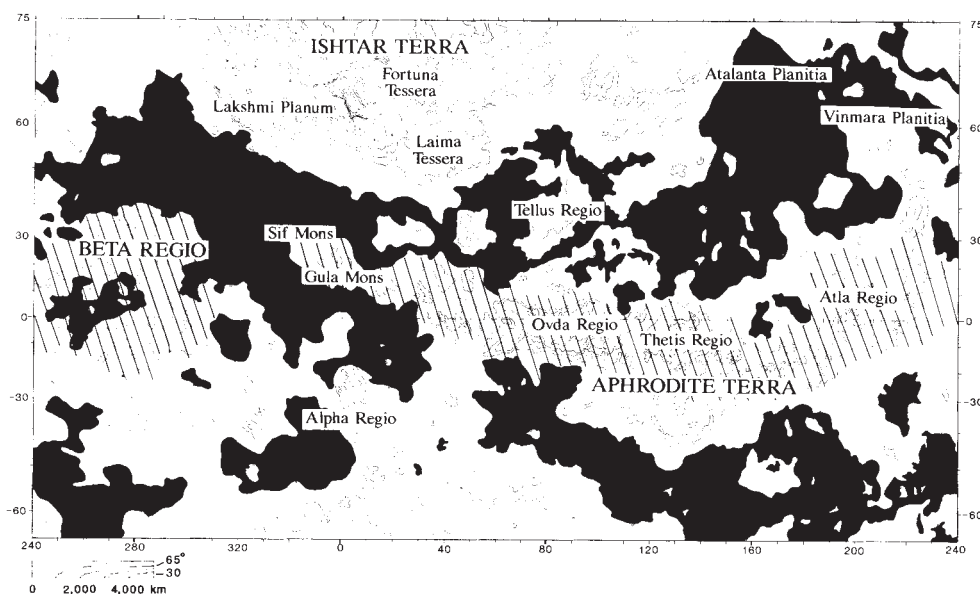


FIG. 1 Global topography and location map of Venus, showing the location of areas described in the text (left). The topography shown is based on VRM Planning Chart, US Geological Survey, 1984. Areas below mean planetary radius (6,051.9) are shown in black and the equatorial highlands are shown cross-ruled. Above, the main highland regions are shown as they appear (in green) on the cover.

apparent depth of compensation¹² of 330 km. On the basis of the relations between geological units and map patterns^{19–22}, the high topography of Beta Regio is interpreted to have formed initially as a result of doming in response to a mantle thermal anomaly (hotspot), accompanied by localized volcanism to produce Rhea Mons. Continued uplift caused extension and the formation of a rift zone (Devana Chasma) splitting and separating Rhea Mons. Volcanism accompanied rifting, forming Theia Mons, a shield volcano superposed on the western bounding fault, partly filling the rift zone and producing flanking deposits which form a veneer on the broad rise adjacent to the rift zone. Devana Chasma bifurcates in the vicinity of Theia Mons^{23,24}, with rift zones extending south to Phoebe Regio and west toward Atla Regio and Aphrodite Terra, giving the region of southern Beta the appearance of a tectonic junction²³ (Fig. 2a). Geological observations and map patterns of faults suggest that Beta is not simply a passive rift linked to domal uplift, but that active lithospheric stretching has been involved^{19,24}. Early analyses^{17,25} and more recent data^{22,26} indicate that flanking plateaus possess radar and morphological characteristics similar to tessera terrain²¹, a tectonically deformed terrain consisting of linearly arrayed troughs and ridges. The density of impact craters suggests that Beta Regio¹⁹ is geologically young, comparable to the age of the northern high latitudes²⁷. Venera lander data indicate a basaltic composition for surfaces in the Beta-Phoebe region²⁸.

Beta Regio is apparently the site of a distinctive mantle upwelling (plume or hotspot). That patterns of mantle upwelling may be commonly manifested on Venus is suggested by similarities between Beta and Atla Regiones²³, between these two and the smaller Eisila²² and Bell Regiones²⁹, and by the presence of numerous coronae³⁰ and large shield volcanoes^{22,31}. Thus mantle upwelling in the form of plumes and associated hotspots may be an important factor at several scales in mantle dynamics, in modes of crustal formation and heat loss, and in styles of surface deformation.

Aphrodite Terra. This region extends ~16,000 km along the equatorial highlands³² (Figs 1, 2b). Its crest is characterized by a broad symmetrical rise several thousand kilometres wide and elevations of 1.5–2.0 km in the east and broad plateaus with dimensions of 2,000–2,500 km and altitudes of 3.5–4.5 km in the west (Onda and Thetis Regiones). Central linear troughs (chasmata) 100–200 km wide and 1–2 km deep (such as Dali and Diana) are commonly superposed on the rise crest and are thought to be of extensional origin³³. Distinctive positive gravity anomalies are associated with Aphrodite, although their magni-

tude and apparent depths of compensation are lower than that of Beta^{11,12}. Although no detailed Earth-based images exist, Pioneer-Venus data show that the central plateaus are rough at radar wavelengths, segmented²³, and have many of the characteristics of tessera terrain²⁶, whereas the lower slopes are characterized by volcanic plains. The geochemical data from the Vega landers (Fig. 2b) indicate the presence of basalts³⁴.

The correlation of gravity and topography at long wavelengths and the amplitude of the gravity anomalies^{11,12} have led several workers to suggest that Aphrodite Terra is the site of activity related to mantle plumes or hotspots^{35–38}. A global test for the presence of topography associated with divergent-plate boundaries¹⁰ showed that Aphrodite is comparable to a divergent-plate boundary in that it is linear in extent, and is similarly shaped in cross-section, but that it differs from typical ocean rises in that rise-crest elevations along Aphrodite do not group tightly around a modal elevation, and that the rise does not seem to form part of a globally interconnected system viewed at the present resolution. Analysis of Pioneer-Venus altimetry and imaging, and Arecibo high-resolution altimetry, showed the presence of parallel linear discontinuities extending for several thousand kilometres across Western Aphrodite Terra³⁹. These cross-strike discontinuities separate Western Aphrodite into rectangular blocks or domains 400–500 km wide (Fig. 2b). Topographic profiles parallel to the discontinuities showed evidence for bilateral symmetry across the rise⁴⁰. These linear centres of symmetry are oriented normal to the discontinuities, terminate against them, and are offset in a right-lateral sense between domains (Figs 1, 2b). These characteristics, together with evidence for split and separated topography and topographic step-downs across the discontinuities, consistent with directions of rise-crest offsets, led to the proposal that Western Aphrodite Terra is the site of crustal spreading along a segmented rise crest³⁸. The cross-strike discontinuities seem to be similar to ocean-floor fracture zones, and on the basis of the characteristics of topographic profiles, spreading is occurring at rates of a few centimetres per year^{38,41}. The subsidence of topography away from the rise crests is generally proportional to the square root of distance, consistent with that expected for a divergent thermal boundary layer. For individual profiles, the fit of topography is in general poorer for Venus than for the Earth⁴², but comparable for averaged profiles⁴³. The plateaus lying on the rise (Fig. 2b) may be the result of mantle plumes or hotspots and associated crustal thickening, analogous to Iceland in the North Atlantic³⁸.

Modelling studies of how the environmental conditions on

Venus would affect terrestrial spreading centres⁴³ shows that the major difference between the Earth and Venus would be the influence of the enhanced surface temperature on upper-mantle temperatures on Venus. This would result in increased melting forming thicker crust, and a corresponding increase in the elevation of isostatically compensated topography. Crust on Venus produced at average spreading centres would be ~ 15 km thick⁴³, in contrast to the average oceanic crustal thickness on Earth of 5–7 km. Using the Pioneer-Venus gravity and topography data, this model was then applied to test the hypothesis that Western Aphrodite is a site of crustal spreading³⁸. Topography on the plateau (Fig. 3a,b) is consistent with a spreading half-rate⁴³ of ~ 1.0 cm yr^{-1} . The steep slopes at the edge of the Iceland-like plateau could be explained by the crust being thicker (~ 30 km) in the plateau region. Analysis of the gravity data in a manner similar to that for the topography showed that the maximum gravity values coincide with the ridge axis (the centre of topographic symmetry)⁴³ (Fig. 3c). Elevated upper-mantle temperatures producing enhanced melting and thus thicker crust could account for the observed apparent depth of compensation^{11,37} characterizing this region⁴³. A model consistent with the observed gravity and topography data is one in which a central region with a crust 30 km thick (Ovda Regio, the Iceland-like plateau) has been spreading at ~ 1.0 cm yr^{-1} for the past 200 Myr, preceded by a thinner crust (15 km) now occupying the flanks of Aphrodite. These analyses^{38,43} indicate that Western Aphrodite Terra formed through a combination of hotspots and crustal spreading, both contributing to crustal formation, evolution and heat loss, much like Iceland. Eastern Aphrodite Terra (Fig. 2b) has many of the elements characteristic of Western

Aphrodite Terra (cross-strike discontinuities, broadly symmetrical topography, central troughs, offset rise crests, topographic step-downs), but some elements (such as Iceland-like plateaus) do not occur, the altitude of the rise crest is lower, the magnitude of the gravity anomalies are lower, and the patterns of discontinuities and rise crests are apparently more complex⁴⁴. The broad saddle-shaped region of Eastern Aphrodite shows the best fit to the root-distance relation, yielding apparent spreading half-rates⁴² of a few centimetres per year.

If crustal spreading is occurring in the Aphrodite Terra region at a rate of up to a few centimetres per year, there are consequences that should be testable with available data⁴⁵. These consequences include a young average age for large parts of the planet and a trend in age from younger to older away from the equatorial highlands. Data on the size-frequency distribution of impact craters^{27,46,47} have been interpreted as indicating that the average age of the surface observed so far is < 1 Gyr, and could be < 450 Myr, the average age of Earth's crust^{47,48}. This is consistent with global average ages predicted for a range of spreading rates⁴⁵. For example, it would take ~ 450 Myr for crust created at an equatorial rise crest to travel to 45° N at spreading half-rates of 1 cm yr^{-1} . In addition, a plot of the number of impact craters per unit area from the north pole to $\sim 30^\circ$ N latitude (the limit of the Venera 15–16 coverage) shows a distinct decreasing trend, consistent with a general decrease in age of the surface from the north toward the equator⁴⁵. These observations are consistent with the predictions from crustal spreading in the equatorial regions, although if global crustal spreading is occurring it must be considerably more complex than simple equatorial spreading and polar convergence.

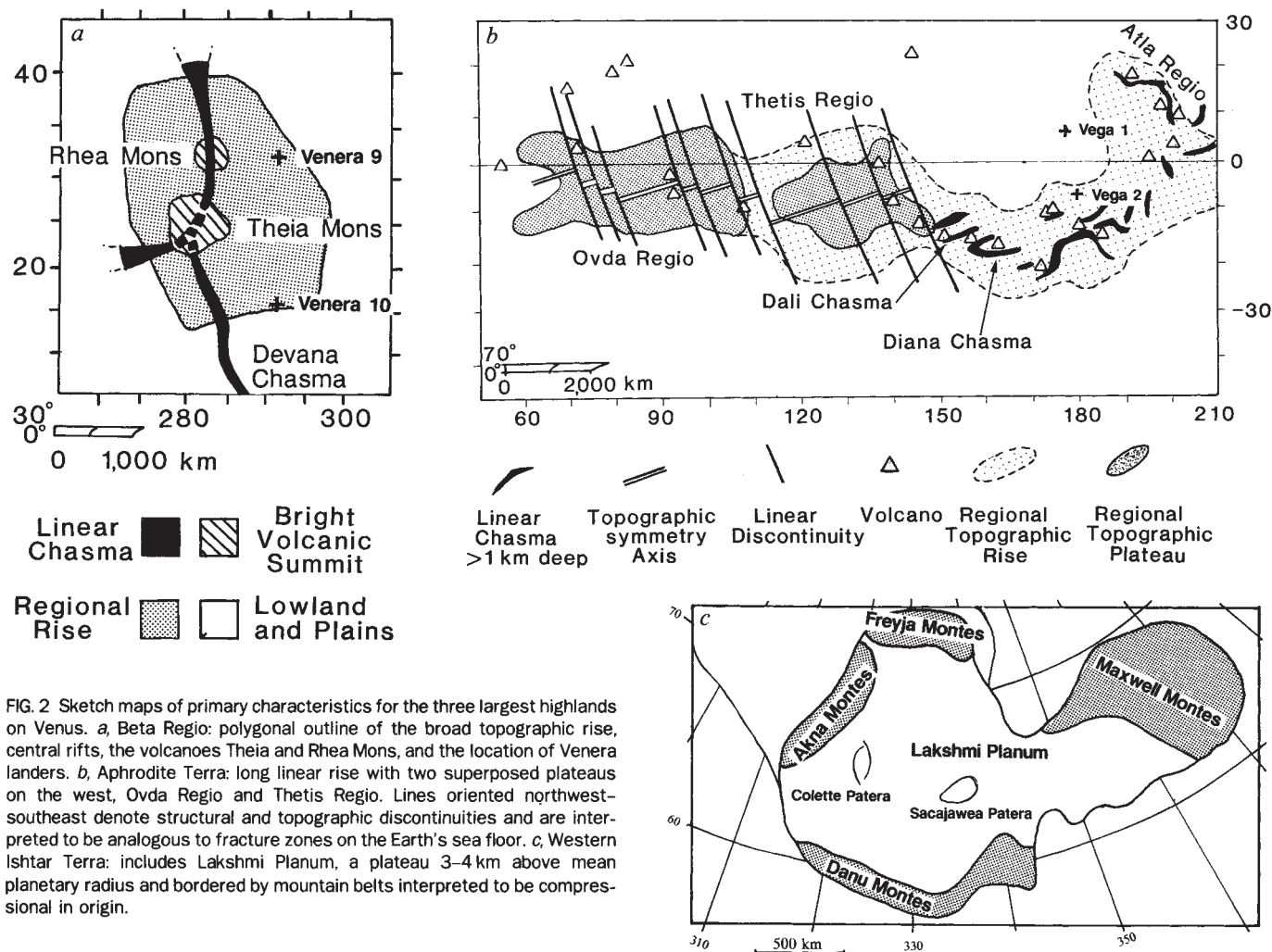


FIG. 2 Sketch maps of primary characteristics for the three largest highlands on Venus. a, Beta Regio: polygonal outline of the broad topographic rise, central rifts, the volcanoes Theia and Rhea Mons, and the location of Venera landers. b, Aphrodite Terra: long linear rise with two superposed plateaus on the west, Ovda Regio and Thetis Regio. Lines oriented northwest-southeast denote structural and topographic discontinuities and are interpreted to be analogous to fracture zones on the Earth's sea floor. c, Western Aphrodite Terra: includes Lakshmi Planum, a plateau 3–4 km above mean planetary radius and bordered by mountain belts interpreted to be compressional in origin.

Ishtar Terra. Covering $\sim 7 \times 10^6 \text{ km}^2$ in the northern high latitudes (Fig. 1), Ishtar Terra is characterized by the steepest bounding slopes of any highland on the planet¹⁸ and differs from the equatorial highlands in many ways. Western Ishtar Terra (Fig. 2c) is dominated by linear mountain belts and some adjacent tesserae surrounding a volcanic highland plain (Lakshmi Planum) with low central volcanoes^{49,50}. Western Ishtar shows evidence for extensive development of convergence and compressional deformation^{51–56} in the form of orogenic belts⁵², adjacent foredeeps of possible flexural origin, and crustal underthrusting^{54,55}. It has an apparent depth of compensation of 130–150 km (refs 57, 58). On the basis of geological observations, Western Ishtar has been interpreted as the result of regional convergence and underthrusting surrounding a pre-existing tessera block⁵⁹, or alternatively, to be the site of an upwelling mantle plume with outward directed forces producing the mountain belts⁶⁰. Eastern Ishtar Terra (Fig. 1) is characterized by tessera terrain of a variety of types and orientations (Fortuna Tessera)^{56,61}. It has been interpreted to be the result of large-scale regional convergence of tessera blocks⁶² and associated deformation and crustal thickening⁵⁶, or alternatively as due to upwelling and gravity sliding^{61,63}.

Intervening regions. The vast majority (>70%) of the terrain seen in the northern mid-to-high latitudes is classified as volcanic plains²¹, which often contain abundant small volcanic shields⁶⁴. The remaining terrain²¹ is characterized by the presence of coronae, ridge belts and regional tectonic deformation. Coronae are circular to oval structures 150–600 km across^{21,30,65} and are thought to be due to rising mantle plumes or hotspots⁶⁵. They occur in a cluster southwest of Ishtar⁶⁶ but are also distributed in other areas^{30,67}. The presence of these features suggests that mantle hotspots may also be present on a smaller scale than that of Beta Regio. Ridge belts are narrow (generally 100–300 km) linear deformed zones^{21,51} having a total length⁶⁸ of 39,000 km, found adjacent to tessera blocks and distributed in abundance in some plains regions⁶⁹. Some workers^{70,71} have interpreted those in Atalanta and Vinmara Planitiae as zones of crustal spreading, whereas others have proposed that many of the ridge belts are of compressional origin^{21,68,69} and that some may be sites of underthrusting and possible subduction⁶⁸. These structures are significant because they contain clues to the amount, distribution and orientation of tectonic strain.

Regional tectonic deformation is seen in 14% of the total area and is characterized by a complex ridged and grooved terrain known as tessera⁵¹, which has three subtypes⁷², sub-parallel-ridged, trough-and-ridge and disrupted. Trough-and-ridge tesserae exhibit a distinctive morphology composed of through-going, generally parallel linear valleys, and perpendicular shorter, narrower valleys and ridges (Fig. 4). This pattern is similar to the generally orthogonal patterns on the terrestrial sea floor of transforms and fracture zones normal to rise crests, and shorter, more closely spaced, abyssal hills parallel to rise crests⁷³. The trough-and-ridge tessera in Laima Tessera⁷⁴ is similar to terrestrial sea-floor structure in terms of linearity, parallelism, length, width, spacing, orthogonality and general morphology. Troughs in Laima Tessera have length, sinuosity and variations in distance between fracture zones in the range of those of the terrestrial sea floor⁷⁵. Trough-and-ridge tessera, however, tend to occur as plateaus at a range of elevations (average $\sim 2 \text{ km}$) above the surrounding plains, whereas terrestrial sea floor ranges in elevation from that observed at rise crests and oceanic plateaus such as Iceland, down to depths of several kilometres on the abyssal floors, where the orthogonal patterns are usually obscured by sedimentation. The plateau nature of the trough-and-ridge tessera could be the result of localized crustal thickening during the spreading process, producing elevated Iceland-like plateaus whose texture is not buried by subsequent lowland volcanic flooding⁷⁴ and is preserved. In areas of larger occurrences of tesserae (such as Fortuna Tessera), the tessera pattern is often more variable and complex⁵⁶, but

zones of trough-and-ridge tessera can be seen with different orientations, suggesting that these large complex areas may be the result of accretion and deformation of plateau-like regions of trough-and-ridge tessera⁶². Other models for the origin of the tesserae⁷² include mantle upwelling and gravity sliding⁷⁶, and deformation associated with crustal thickening⁷⁷.

Geological processes revealed by regional studies

The regional observations indicate that a variety of processes seem to be operating on the surface of Venus. Volcanism is the most areally important geological process, producing extensive volcanic plains²¹, a widespread distribution of small shields⁶⁴, and abundant larger shields and volcanic complexes both in the plains³¹ and associated with extensional deformation (Rhea, Theia, Sif, Gula Montes)^{19,22}. Characteristics of regions such as Beta, the large shield volcanoes and the coronae suggest that mantle upwelling and hotspots are significant on Venus and are widely distributed. Tectonic activity is both widespread and

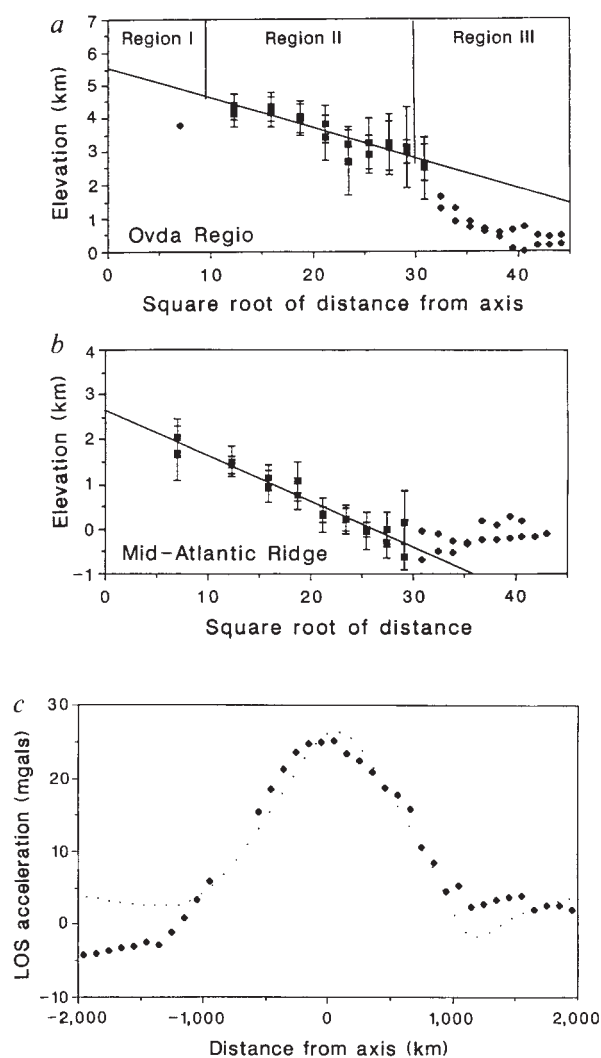


FIG. 3 a, Fit of topography as a linear function of the square root of distance from the centre of Onda Regio (area between the third and fourth linear discontinuities from the left in Fig. 2b). Data points shown represent mean altimetry for distance intervals parallel to the central axis of symmetry. North and south slopes are plotted together. Vertical bars represent standard deviation on mean altitude values. b, Plot similar to a based on bathymetry of Mid-Atlantic Ridge between latitudes 20°N and 40°N. c, Line-of-sight gravity profile (filled diamonds) across Onda Regio compared with calculated gravity profile (dots) for a spreading-centre model using a mantle temperature of 1,600 °C and half-spreading rate of 0.5 cm yr⁻¹ (after Sotin *et al.*⁴³).

localized. Evidence has been presented for extension in the nearly globe-encircling equatorial highlands³³ and crustal spreading³⁸ in the 16,000-km-long Aphrodite Terra region (Figs 1, 2b). Zones of convergence, underthrusting and possible crustal loss, distinctive orogenic belts, and possible hotspots have been identified in the Ishtar Terra region^{49,51–54} (Fig. 2c). Linear deformation zones (ridge belts) with a cumulative length of ~39,000 km have been mapped in the 25% of the surface viewed by Venera 15 and 16⁶⁸. Venera 15 and 16 also revealed that ~10–15% of the area is characterized by patches of tessera terrain surrounded by volcanic plains⁶⁷. Several plateaus of tesserae are characterized by a sea-floor-like, abyssal hill/fracture zone fabric. Other tessera regions may represent convergence of crustal blocks and associated crustal thickening (Fortuna Tessera)^{56,62}, gravity sliding^{61,76} or patterns of deformation associated with ductile crustal thickening⁷⁷. Impact cratering is not an areally or volumetrically significant geological process²⁷ and the age of the surface^{27,47} is more comparable to that of the Earth⁴⁸ (<1 Gyr) than to that of the Moon, Mars or Mercury (>3 Gyr).

Crustal spreading/mantle plume model

From the characteristics of Venus outlined above, it is clear that mantle plumes are forming at a variety of scales, ranging from coronae (200–600 km) to Beta and Atla Regio (2,000–3,000 km). The larger plumes seem to be preferentially localized in the equatorial highlands, and some may be linked directly to crustal spreading processes. On the basis of these observations and interpretations, we propose a model (the crustal spreading/mantle plume model) in which crustal spreading and superposed Iceland-like mantle plumes associated with the crustal spreading process⁴⁰ dominate at least one-third of the Equatorial Highlands (Figs 1, 5) and may account for many of the regional and global characteristics outlined above.

Crustal spreading. In this model, Aphrodite Terra is a site of crustal spreading in a direction normal to the rise crest. The bilaterally symmetrical topography is segmented in an echelon manner into different domains by transform and fracture-zone-like cross-strike discontinuities (Fig. 2b). Crust about 15 km thick is created and moves laterally away from the rise⁴³, cooling and descending into the lowlands to become modified by subsequent volcanism and tectonism (Fig. 5). Topographic step-downs are often observed in the lowlands along the extensions of the fracture-zone-like features, and the sense and magnitude of the step-down is generally consistent with that predicted from the observed rise-crest offsets⁴¹. Topographic profiles (Fig. 3) suggest spreading rates^{38,41–44} of the order of 1–3 cm yr⁻¹.

Mantle plumes and the formation of 'plume plateaus'. From time to time, mantle upwelling produces plumes and hotspots beneath the spreading axis similar to the situation that exists at Iceland today⁷⁸. These hotspots cause increased upper-mantle temperatures (~100 °C) below the rise crest, and increased melting and crustal production; a thicker crust (~30 km) is formed, with an increase in isostatically supported elevation of ~1.5 km. As with Iceland, the influence of this hotspot over time creates a plateau of thicker crust astride the spreading centre. Localized plateaus in Aphrodite Terra, such as Ovda and Thetis Regiones, are thought to be the site of such hotspot activity in a crustal spreading environment^{38,41,43,45} (Fig. 5). The fact that the peak gravity values correspond in location to the rise crest⁴³ suggests that the hotspot is closely linked to spreading. The size of Ovda Regio (2,000 km wide) coupled with the spreading rate suggest that this mantle plume has been active for the past 200 million years⁴³.

Evolution of 'plume plateaus'. If the effects of the hotspot waned, the enhanced temperature of the upper mantle below the newly created plateau would return to normal. Crustal spreading would continue, but correspondingly thinner crust (~15 km instead of 30 km) would be produced and isostatically compensated

topography would be 1–1.5 km less than during the period of thicker-crust formation. In this case, continued spreading in the newer, thinner crust, would split the plume-induced plateau, separate it, and move it laterally off the thermal rise into the adjacent lowlands⁷⁹ at rates comparable to the spreading rates (Fig. 5). Although the split and separated plateau would become topographically lower as it cooled, the plateau remains elevated above the adjacent surrounding plains because of its enhanced crustal thickness and isostatic compensation. Some of the plateaus in the northern high latitudes characterized by the sea-floor-like trough-and-ridge tesserae (such as Laima Tessera⁷⁴) are thus interpreted to be 'plume plateaus', remnants of thick crust originally created at rise crests and transported laterally to their present positions.

Relationship to gravity data and sub-surface processes. Terrestrial sea-floor topography is strongly correlated with gravity at shorter wavelengths, but the apparent depth of compensation (ADC) beneath normal terrestrial spreading centres⁸⁰ seems to be too shallow to explain areas such as Western Aphrodite in terms of simple crustal spreading. Variations in the apparent depth of compensation along the equatorial highlands are also observed, with values ranging from 50 km to 250 km between Western Aphrodite and Atla Regio³⁷. There are also correlations between characteristic gravity values and style of geological structure^{23,81} in the equatorial highlands, with the largest values for ADCs^{12,37} coinciding with the tectonic junctions^{23,81} at Beta and Atla Regiones. Most workers conclude that the characteristics and magnitudes of the ADCs of the equatorial region require dynamic support mechanisms, and mantle upwelling or hotspots are common interpretations of these data^{12,35–37,81}.

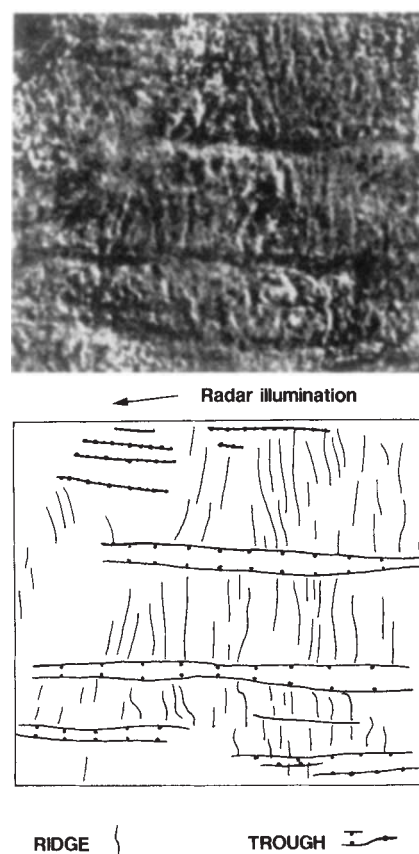


FIG. 4 Venera 15 and 16 synthetic aperture radar image of a portion of Laima Tessera (top). Long linear troughs and short discontinuous ridges orientated approximately at right angles may be analogous to patterns associated with sea-floor (fracture zone/abyssal hill) spreading fabrics on Earth⁷⁴.

Our model accounts for the observed gravity data by a combination of crustal spreading and mantle upwelling in the following way (Fig. 5): normal crustal spreading on Venus is characterized by buoyant rise of mantle material at relatively shallow depths, and correlation of gravity and thermal topography at relatively short wavelengths. From time to time, plumes rise from lower mantle depths below the spreading centre. Their evolution is governed by their low-viscosity low-Reynolds-number fluid dynamical behaviour in a convecting viscous medium⁸². In general, the buoyant diapirs ascend as spherical blobs which decelerate, collapse and flow out laterally as they approach the viscous lid. The ascending plumes begin to influence the crustal spreading environment through thermal uplift and increased melting to produce thickened crust. As the plume head impinges on the viscous lid and flows out laterally, an Iceland-like 'plume plateau' of thickened crust forms (Fig. 5). As the plume head collapses and decays, the plateau continues to grow and move laterally through crustal spreading processes under the influence of the plume tail until the region below the spreading centre returns to normal upper-mantle temperatures. After this, thin (normal) crust is again produced at the spreading centre, and the 'plume plateau' of thickened crust is split and moved laterally away (Fig. 5). These various stages would be characterized by different apparent depths of compensation, contributions to which include dynamic factors (depth, temperature and geometry of the mantle plume), and static factors (variations in crustal thickness). Outside of the equatorial highlands the correlation of gravity and topography and the magnitude of the gravity anomalies is often not as great^{11,83}. For example, some of the plateaus of tessera terrain (such as Tellus, Laima and Alpha) seem to be characterized by small anomalies and relatively shallow depths of compensation^{11,83}. These examples are consistent with the shallow compensation expected from Iceland-like 'plume plateaus' of thickened crust that were formed at rise crests and moved laterally off the rise to adjacent lowlands away from the effects of the hotspot (Fig. 5).

Crustal evolution. Crust formed by crustal spreading and moved laterally away from spreading centres should be relatively constant in thickness (~15 km) except where 'plume plateaus' are formed at hotspots. Spreading rates in the range of 1–3 cm yr⁻¹ predict a relatively young average age for the surface, well within the range observed^{27,46,47}. Processes of crustal loss must be occurring if crust is being formed and moved laterally at these rates. Previous analyses⁵ indicated that the basalt-eclogite

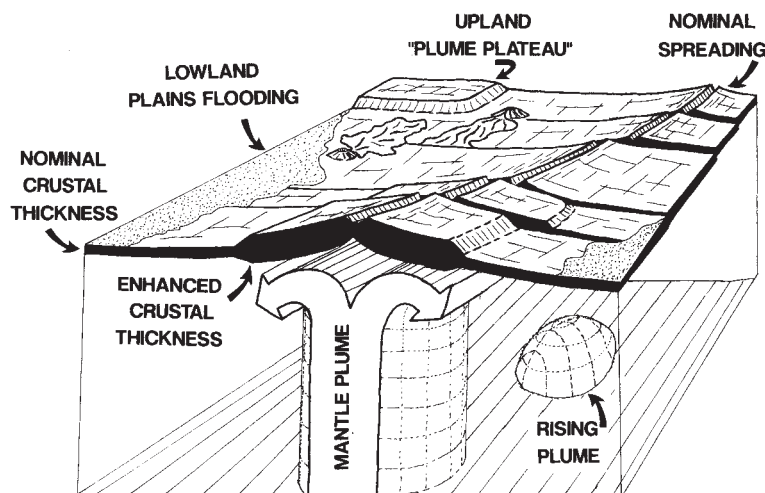
transition causing densification would be deeper than on Earth, and the thick basaltic crust would be less dense, more buoyant and non-subductable. More recent assessments^{84,85} treat cooling thermal boundary layers as a function of crustal thickness (Fig. 6) and show that thin crust (<20 km) becomes negatively buoyant and thus would be susceptible to subduction in <250 Myr or within a few thousand kilometres of a spreading centre. Regions of compressional deformation⁵⁴ and orogenic belts⁵² with adjacent foredeeps of possible flexural origin⁵⁵ may therefore be sites of crustal underthrusting, subduction and crustal loss.

On the basis of the plume-plateau formation mechanism⁴³ and estimates of crustal thickness of tesserae from Airy isostasy⁴, plateaus of tessera terrain interpreted to be candidates for 'plume plateaus' represent crustal thicknesses in the range 20–50 km. In this thickness range, plume plateaus would be predominantly positively buoyant (Fig. 6), and would tend to resist subduction. Larger accumulations of complex tesserae (such as Fortuna Tessera) may represent accretion and deformation of several smaller plateaus of thicker crust that have undergone convergence and deformation^{56,86} and possibly suturing of buoyant crustal blocks into 'proto-continental' regions⁶².

Lack of information about the detailed nature of tectonic zones over the whole of Venus precludes the determination of the global distribution of the different types of boundaries that may be associated with crustal spreading and crustal loss and the total length and significance of this process, although Aphrodite Terra alone is about one-third of the total length of crustal spreading centres observed on Earth at present. Simple geometric considerations, as well as consideration of poles of rotation for Aphrodite Terra⁴², indicate that spreading must be more complex than simple equatorial extension and poleward spreading. Complex boundaries and histories may be encountered away from Aphrodite, in a manner similar to the older parts of the Pacific Plate between the East Pacific Rise and the subduction zones of the Western Pacific.

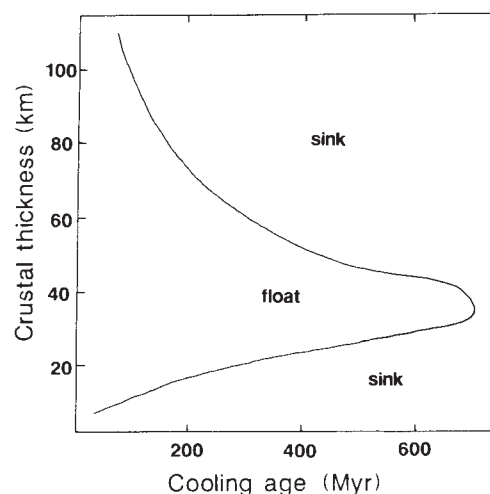
The presence of the equatorial highlands, their dominance of the global topography⁸⁷ (Figs 1, 7), the characteristics of the gravity data, and the large number of apparent mantle plumes and plume plateaus there relative to the Earth suggest that the crustal spreading processes seen in Aphrodite Terra may be more closely linked to large-scale mantle upwelling than is the case on Earth. Specifically, we would characterize terrestrial crustal spreading as relatively 'passive' with respect to large-scale mantle convection, with crust being formed at relatively transient

FIG. 5 Block diagram showing the general characteristics of the crustal spreading/mantle plume model. The relative thickness of the crust is shown in black; structure of the lithosphere is not shown. In areas of nominal spreading (background), normal Venus upper-mantle temperatures (higher than those on Earth) produce crust with a thickness of ~15 km (ref. 43). Where spreading occurs over areas of deep mantle upwelling (mantle plumes) (foreground) upper-mantle temperatures are higher, melting is enhanced, and the crustal thickness produced during spreading is increased relative to that produced during nominal spreading conditions. Continued spreading at the rise crest produces Iceland-like plateaus (plume plateaus) of thickened crust (typical thicknesses might be 30 km; ref. 43). When the temperature decreases and the upper mantle temperature returns to normal beneath the spreading centre, the thickness of the crust produced is diminished. The plume plateau is then split and separated, and carried laterally on the diverging halves of the lithosphere into the lowlands. As the lithosphere cools and subsides, the plateau eventually attains an isostatic elevation somewhat greater than the surrounding lowlands (upland plume plateau) (background) owing to its greater crustal thickness. The tectonic fabric associated with spreading may be buried locally by subsequent off-axis volcanism. Plumes wax and wane both vertically and



along strike in the equatorial highlands to produce variable rise-crest characteristics and gravity signatures, and complex relationships between local and regional apparent depth of compensation and geological structure.

FIG. 6 Buoyancy of the thermal lithosphere of Venus as a function of crustal thickness and lithospheric cooling age⁸⁵. After ~200 million years, crust of <20 km thickness is negatively buoyant and susceptible to subduction. Most crust on Venus is thought to be <20 km thick^{93,94}. Regions with crustal thickness in the range 25–50 km (comparable to estimated tessera and mantle-plume crustal thicknesses^{4,43}) remain buoyant and their subduction is inhibited.



zones of localized upwelling at spreading centres (the positions of which change over the course of tens to hundreds of millions of years owing to ridge jumps and absolute plate movement), and moving laterally as part of the evolving lithospheric thermal boundary layer partly detached from the deeper mantle by the asthenosphere. The system is driven largely by the negative buoyancy of the descending lithospheric slabs, and deeper mantle convection produces large swells that are not linked directly to spreading processes, or more-localized hotspots which coincide with a spreading centre only occasionally (as in the case of Iceland). For Venus we hypothesize that in several parts of the equatorial highlands (Beta, Atla, Ovda, Thetis and perhaps Eisila) upwelling may be more dynamic, and thus crustal spreading may be linked more closely to large-scale mantle upwelling than on Earth (many Iceland's along the axis of crustal spreading, rather than just one). The geological and thermal boundary layer characteristics of over one-third of the topography, the magnitude and variability of the gravity anomalies, and the variation of gravity, topography and geology along the highlands lead to the proposal that the equatorial highlands represent the result of a series of broad mantle plumes that are common to the near-equatorial region, but variable in time and longitude. The cause of this variability is not known but a plausible explanation is that it represents a series of upwellings that are at different stages of ascent and evolution (Fig. 5), such as hotspot heads and tails⁸⁸. The source of these upwellings, their detailed role in and link to crustal spreading, and the cause of their apparent linearity is unknown, but may be linked to stages in the thermal evolution of the mantle⁸⁹ or to the structure and evolution of the core-mantle boundary.

Heat loss, crustal formation and tectonic style

Heat loss across the outer thermal boundary layer (the lithosphere)² seems to take place largely by conduction on Venus^{90,91}, whereas on Earth about 62% of the heat loss occurs by plate recycling. Surface volcanic deposits, although areally extensive, are apparently emplaced at low enough rates⁹¹ that they are not a significant contribution to heat loss through advection. Crustal spreading and recycling, although potentially important as a mechanism of crustal formation and surface deformation, apparently occur at such low rates^{38,41–43} that even if all of the 36,000-km-long equatorial highlands were spreading (compared with the 60,000 km effective length of spreading centre on Earth), the heat loss would be largely by conduction in older parts of the spreading crust and lithosphere. Crustal spreading could be operating on Venus as on Earth as the principle crustal formation mechanism, but contributing <15% to the global heat loss for Venus, only about one-fourth of the heat loss associated with crustal spreading on Earth.

Crustal formation processes are not well constrained⁴,

although the majority of the crust seems to be secondary³ (derived from mantle partial melting). Estimates of Venus crustal thickness are 10–20 km for most of the surface^{92–94} and, on the basis of Airy isostasy, may be locally higher in the tesserae⁴ and the mountain belts⁵⁶. Estimates of total crustal volume are similar to that for the present Earth^{4,93}. If this volume represents all the crust that Venus has produced during its entire history, then Venus is producing crust at extremely low rates by terrestrial standards⁹³. In this case, some of the primary³ crust (produced by accretional heating) may still be preserved at the base of the present crust. The Earth has produced many times its present crustal volume over its history, and if Venus is also recycling its crust the present volume is not the total volume produced over time on Venus⁴—there is 'missing crust'. Tertiary crust³ (produced by reworking of secondary crust) may be forming in the orogenic belts or tesserae^{4,56,95} on Venus, in a manner similar to that on terrestrial continents.

An important question is the general tectonic style and mechanisms of tectonic deformation on Venus. Gravity anomalies are positively correlated with topography at long wavelengths in the equatorial region¹¹ and have been interpreted to show variable apparent depths of compensation within the highlands^{12,35–37}. Local regions of high-amplitude anomalies (such as Beta and Atla Regio), where the apparent depth of compensation is large, suggest the presence of some type of dynamic support, such as large-scale mantle upwelling or mantle plumes^{12,35–37}. Geological evidence exists for mantle upwelling, extension and rifting, crustal spreading, compression and underthrusting, and regional deformation. This suggests both local mantle upwelling (hotspots) and associated deformation, as well as large-scale regional extension (equatorial highlands), compression (Ishtar Terra and possibly many of the ridge belts), and possible large-scale horizontal movement and deformation of the crust. There is evidence in the northern hemisphere for a latitudinal variation in styles of tectonism and volcanism, with the equatorial region characterized by extensional deformation^{23,33} and the northern high latitudes (particularly Ishtar Terra) characterized by compressional deformation⁵². Major questions remain concerning tectonic style. Which model—individual hotspots and limited lateral movement, or crustal spreading and associated hotspots—can best account for the observed geological and geophysical characteristics? How are mantle dynamics⁹⁶ and mantle flow linked to surface deformation? What is the influence of a ductile lower crustal layer^{76,77,94} on the deformation of the crust and lithosphere, and how much of the observed deformation is linked to this phenomenon through processes such as ductile crustal thickening and thinning⁷⁷, and gravity sliding⁷⁶?

Does Venus have plate tectonics? On Earth, the thermal structure is such that the outer thermal boundary layer (the lithosphere)

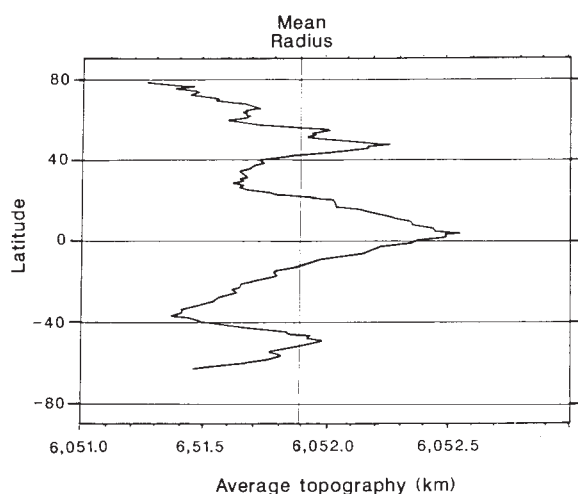


FIG. 7 Average surface elevation of Venus as a function of latitude relative to an equator running through the equatorial highlands and inclined $\sim 20^\circ$ to the equator. At a global scale the topography of Venus is dominated by the equatorial highlands and consists of an equatorial high bordered by two lowland troughs and two polar highs. The large scale and symmetry of this global topographic pattern suggest that it may be related to the fundamental geometry of convection in the deep mantle.

is cool enough that it remains rigid and does not significantly deform over long timescales, whereas the higher temperatures in the mantle permit solid-state creep and fluid-like behaviour over similar timescales. Plate tectonics describes the creation, lateral movement and loss of rigid lithospheric plates. The rigidity of the plates implies that they transmit elastic stresses over geological time, that they act as stress guides capable of transmitting stress over great distances, and that stretching, distortion and folding do not occur significantly within the plate. The rigidity, which ensures the lack of significant internal deformation within the plates and the predictability of plate movements, is a key factor in plate tectonics. Because the Earth is a sphere with essentially fixed surface area, spreading velocities at ridges can be related to subduction velocities, relative velocities between rigid plates can be determined, and, using Euler's theorem, the relative motions between two adjacent plates can be specified relative to poles of rotation.

On Venus, evidence suggests that conditions may not be favourable for such rigid Earth-like plate tectonics, even in an environment of crustal spreading, lateral movement and subduction. First, the very high surface temperature on Venus means that the thermal gradient will be such that the lower crust of Venus may be a relatively low-strength layer⁹². Thus, lithospheric plates on Venus may be poorer stress guides and deformation may be less focused at plate boundaries, and more diffuse throughout the plate⁴². This situation is similar to that observed in the upper crust of continents on Earth⁹⁷, which ride relatively passively on lithospheric plates. Owing to compositional differences, the continents are weaker and more easily deformed than oceanic crust. This factor, and the presence of a low-strength deeper crustal layer in the continents, enhances internal deformation, and the predictability of movement characteristic of the rigid oceanic plates is lost.

Second, positive correlation of gravity and topography at long wavelengths on Venus has been interpreted to mean that a discrete asthenosphere (the partially molten layer at the base of the lithosphere) may be lacking on Venus³⁵ and that surface movement may be linked more directly to mantle flow⁹⁶. In this case, crustal spreading could occur and be a major process of crustal formation, but the balance of forces associated with lithospheric movement⁹⁸ would be such that the lithosphere would be much more susceptible to deformation associated with mantle dynamics, and the predictability of the movement of a relatively detached rigid plate would be lost. Thus, it is possible that even in a crustal spreading environment on Venus, rigid-plate kinematics characteristic of terrestrial oceanic plate tectonics may not describe the movement of the lithosphere, particularly in reference to the predictability of movement of lithospheric plates relative to one another. Crustal spreading on Venus may be like the Earth's oceanic crust in the spreading

sense, but more like the Earth's continents in the sense of the subsequent deformational history (plastic-plate tectonics rather than rigid-plate tectonics). Further analysis of the hypothesized boundaries, their geometric characteristics and distribution of deformation is required to address this question.

Goals, analyses and predictions for Magellan

As the Magellan spacecraft circumnavigates Venus in an elliptical near-polar orbit¹⁶, the planet will rotate beneath it. In one Venus day (243 Earth days) (cycle 1: August 1990 to April 1991) and in subsequent cycles, the radar experiment will obtain radar images of virtually the entire surface at a resolution of several hundred metres. Data on the surface characteristics (roughness, reflectivity, radiometry) will also be acquired. Altimetry data will be obtained simultaneously at a resolution an order of magnitude greater and with a footprint a factor of three smaller than previous data. In later cycles, once a global imaging and altimetry data set are obtained, the unique capabilities of the radar instrument will be used to obtain stereo images, interferometric data, even higher-resolution altimetry, and to search for changes on the surface such as new lava flows. Doppler tracking of the spacecraft will provide improved gravity data and in the later stages of the mission it may be feasible to make the orbit circular and to obtain gravity data with a horizontal resolution of several hundred kilometres. Magellan will thus provide image coverage of extremely high resolution over the entire globe (a data set better than we have for the Earth's ocean floor!), and much-improved topography and gravity coverage and resolution. This will be a remarkable step in planetary exploration comparable to stripping the ocean cover off the sea floor (two-thirds of the Earth's surface) and providing scientists with complete high-resolution data in less than one Earth year.

This perspective underlines the fact that Magellan is first and foremost a voyage of exploration. Magellan scientists are first trying to understand how different processes will manifest themselves in landforms in the Venus environment so that the global images will be correctly interpreted. For example, it is known that the very high atmospheric pressure at the surface of Venus will inhibit gas exsolution of rising magma and prohibit explosive volcanic eruptions except under certain circumstances⁹⁹. What about other processes? Does the high surface temperature change the rigidity of the outer deforming layer so much that laterally moving crust and lithosphere will look different from the Earth's lithospheric plates? If elevated temperatures cause the ratio of extrusion to intrusion of magma to change, will zones of crustal spreading on Venus look like the spreading centres of the Earth's oceans?

Second, the exploration aspect of the mission means that there will be an extended period of time during which regional

analyses of what has previously been *terra incognita* will be undertaken, and these results will be compiled and synthesized into a global picture of the geology and geophysics of Venus. This may take some time, considering that several hundred years of exploration and synthesis of Earth geology was required before a global understanding emerged in 1960s. These studies and syntheses will be addressing several critical questions. What is the global distribution of volcanoes and which have been active recently? How are they linked to the loss of heat and formation of the crust? What is the global distribution of zones of extension and compression? Are they isolated around mantle upwellings and hotspots, or do they form mosaic-like patterns similar to the Earth's tectonic plates? On the basis of impact crater density, what is the age of the surface? Are there any very ancient terrains?

Third, high-resolution data will permit detailed analysis of specific questions raised by previous studies. What do flexural signatures around volcanoes say about regional lithospheric thickness and thermal gradient? What do the high-resolution data reveal about the extensional or compressional nature of ridge belts? What is the role of tessera formation in environments of mantle upwelling such as Beta Regio? Does Atla Regio represent mantle upwelling as Beta Regio appears to? How

many other large upwellings presently occur on Venus? How widespread is the correlation of gravity and topography, and what is the geological context for different apparent depths of compensation?

Finally, in some cases Magellan data will permit specific tests of predictions of current hypotheses. For example, if crustal spreading is occurring in Aphrodite Terra, then symmetry in ages and features should be observed about the axis of spreading. If Iceland-like crustal spreading is producing 'plume plateaus' in Western Aphrodite Terra, and if the trough-and-ridge tessera (as seen at Laima Tessera) represents 'plume plateaus' that have been split and separated from a spreading centre, then Magellan data should reveal trough-and-ridge tessera patterns on the plateaus of Western Aphrodite. If plate tectonics occurs on Venus, are the plates rigid or plastic? These varied investigations of Venus, the most Earth-like of the terrestrial planets, will provide fundamental information on processes of crustal formation and evolution, modes of surface deformation and heat loss, and mantle dynamics. This emerging picture will also surely provide new insight to the Earth's interior and history. □

J. W. Head and L. S. Crumpler are at the Department of Geological Sciences, Brown University, Providence, Rhode Island 02912, USA.

1. Head, J. W. & Solomon, S. C. *Science* **213**, 62-76 (1981).
2. Solomon, S. C. & Head, J. W. *J. geophys. Res.* **87**, 9236-9246 (1982).
3. Taylor, S. R. *Tectonophysics* **161**, 147-156 (1989).
4. Head, J. W. *Earth Moon & Planets* (in the press).
5. Anderson, D. L. *Geophys. Res. Lett.* **8**, 309-311 (1981).
6. Pettengill, G. H. et al. *J. geophys. Res.* **85**, 8261-8270 (1980).
7. Masursky, H. et al. *J. geophys. Res.* **85**, 8232-8260 (1980).
8. Morgan, P. & Phillips, R. J. *J. geophys. Res.* **88**, 8305-8317 (1983).
9. Kaula, W. M. & Muradian, L. M. *Geophys. Res. Lett.* **9**, 1021-1024 (1982).
10. Kaula, W. M. & Phillips, R. J. *Geophys. Res. Lett.* **8**, 1187-1190 (1981).
11. Sjogren, W. M. et al. *J. geophys. Res.* **88**, 1119-1128 (1983).
12. Esposito, P. B. et al. *Icarus* **51**, 448-459 (1982).
13. Campbell, D. B. et al. *Science* **221**, 644-647 (1983).
14. Jurgens, R. F. et al. *J. geophys. Res.* **85**, 8282-8294 (1980).
15. Aleksandrov, Yu. N. et al. *Soviet Sci. Rev E6*, 61-100 (1988).
16. Saunders, R. S. et al. *J. geophys. Res.* **95**, 8339-8355 (1990).
17. Head, J. W. et al. *J. geophys. Res.* **90**, 6873-6885 (1985).
18. Sharpton, V. L. & Head, J. W. *J. geophys. Res.* **90**, 3733-3740 (1985).
19. Stofan, E. R. et al. *Geol. Soc. Am. Bull.* **101**, 143-156 (1989).
20. Campbell, D. B. et al. *Science* **226**, 167-170 (1984).
21. Barsukov, V. L. et al. *J. geophys. Res.* **91**, 378-398 (1986).
22. Campbell, D. B. et al. *Science* **246**, 373-377 (1989).
23. Senske, D. A. *Earth, Moon & Planets* (in the press).
24. McGill, G. et al. *Geophys. Res. Lett.* **8**, 737-740 (1981).
25. Basilevsky, A. T. et al. *Moon & Planets* **27**, 63-89 (1982).
26. Bindshchadler, D. L. et al. *Geophys. Res. Lett.* **17**, 171-174 (1990).
27. Ivanov, B. A. et al. *J. geophys. Res.* **91**, 413-430 (1986).
28. Surkov, Yu. A. et al. *J. geophys. Res.* **89**, 393-402 (1984).
29. Janie, P. D. J. & Basilevsky, A. *Earth, Moon & Planets* **39**, 251-273 (1987).
30. Pronin, A. A. & Stofan, E. R. *Icarus* (in the press).
31. Slyuta, E. N. *Lunar planet. Sci.* **21**, 1172-1173 (1990).
32. Phillips, R. J. & Malin, M. C. in *Venus* (eds Hunten, D. H. et al.) 159-214 (Univ. Arizona Press, Tucson, 1983).
33. Schaber, G. G. *Geophys. Res. Lett.* **9**, 499-502 (1982).
34. Surkov, Yu. A. et al. *J. geophys. Res.* **91**, 215-218 (1986).
35. Kiefer, W. S., Richards, M. A. & Hager, B. H. *Geophys. Res. Lett.* **13**, 14-17 (1986).
36. Bills, B. G., Kiefer, W. S. & Jones, R. L. *J. geophys. Res.* **92**, 10335-10351 (1987).
37. Herrick, R. R., Bills, B. G. & Hall, S. A. *Geophys. Res. Lett.* **16**, 543-546 (1989).
38. Head, J. W. & Crumpler, L. S. *Science* **238**, 1380-1385 (1987).
39. Crumpler, L. S., Head, J. W. & Harmon, J. K. *Geophys. Res. Lett.* **14**, 607-610 (1987).
40. Crumpler, L. S. & Head, J. W. *J. geophys. Res.* **93**, 301-312 (1988).
41. Crumpler, L. S. & Head, J. W. *Tectonophysics* (in the press).
42. Grimm, R. & Solomon, S. C. *J. geophys. Res.* **94**, 12103-12131 (1989).
43. Sotin, C. et al. *Earth planet. Sci. Lett.* **95**, 321-333 (1989).
44. Crumpler, L. S. & Head, J. W. *Lunar planet. Sci.* **19**, 233-234 (1988).
45. Head, J. W. & Crumpler, L. S. *Earth Moon & Planets* **44**, 219-231 (1989).
46. Campbell, D. B. & Burns, B. A. *J. geophys. Res.* **85**, 8271-8281 (1980).
47. Schaber, G. G., Shoemaker, E. & Kozak, R. C. *Solar System Res.* **21**, 144-151 (1987).
48. Sclater, J. G., Jaupart, C. & Galsdon, D. *Rev. Geophys. Space Phys.* **18**, 269-312 (1980).
49. Pronin, A. A. et al. *Solar System Res.* **20**, 53-63 (1986).
50. Roberts, K. M. & Head, J. W. *Earth Moon & Planets* (in the press).
51. Basilevsky, A. T. et al. *J. geophys. Res.* **91**, 399-411 (1986).
52. Crumpler, L. S., Head, J. W. & Campbell, D. B. *Geology* **14**, 1031-1034 (1986).
53. Vorder Bruegge, R. W., Head, J. W. & Campbell, D. B. *J. geophys. Res.* **95**, 8357-8381 (1989).
54. Head, J. W. *Geology*, **18**, 99-102 (1990).
55. Solomon, S. C. & Head, J. W. *Lunar planet. Sci.* **20**, 1030-1031 (1989).
56. Vorder Bruegge, R. W. & Head, J. W. *Geophys. Res. Lett.* **16**, 699-702 (1989).
57. Sjogren, W. L., Bills, B. G. & Mottinger, N. A. *Geophys. Res. Lett.* **11**, 489-491 (1984).
58. Grimm, R. E. & Phillios, R. J. *Lunar planet. Sci.* **21**, 437-438 (1990).
59. Roberts, K. A. & Head, J. W. *Lunar planet. Sci.* **21**, 1021-1022 (1990).
60. Pronin, A. A. *Geotectonics* **20**, 271-280 (1986).
61. Sukhanov, A. V. *Geotectonics* **20**, 294-305 (1986).
62. Vorder Bruegge, R. W. & Head, J. W. *Earth Moon & Planets* (in the press).
63. Kozak, R. C. & Schaber, G. G. *Geophys. Res. Lett.* **16**, 175-178 (1989).
64. Aubele, J. C. & Slyuta, E. N. *Earth Moon & Planets* (in the press).
65. Stofan, E. R. et al. *J. geophys. Res.* (in the press).
66. Stofan, E. R. & Head, J. W. *Icarus* **83**, 216-243 (1990).
67. Sukhanov, A. L. et al. *Map 1-2059* (US Geological Survey, 1989).
68. Frank, S. & Head, J. W. *Earth Moon & Planets* (in the press).
69. Kryuchkov, V. P. *Lunar planet. Sci.* **19**, 649-650 (1988).
70. Sukhanov, A. L. & Pronin, A. A. *Dokl. Akad. Sci. USSR* **294**, 661-665 (1987).
71. Sukhanov, A. L. & Pronin, A. A. *Proc. lunar planet. Sci. Conf.* **19**, 335-348 (1989).
72. Bindshchadler, D. L. & Head, J. W. *J. geophys. Res.* (in the press).
73. Macdonald, K. C. & Luyendyk, B. *Mar. geophys. Res.* **7**, 515-535 (1985).
74. Head, J. W. *J. geophys. Res.* **95**, 7119-7132 (1990).
75. deCharon, A. V. & Head, J. W. *Bull. Am. astr. Soc.* **21**, 921 (1989).
76. Smrekar, S. & Phillips, R. J. *Geophys. Res. Lett.* **15**, 693-696 (1988).
77. Bindshchadler, D. B. & Parmentier, E. M. *Lunar planet. Sci.* **20**, 78-79 (1989).
78. Schilling, J. G. *Nature* **242**, 565-571 (1973).
79. Crumpler, L. S. & Head, J. W. *Lunar planet. Sci.* **19**, 235-236 (1988).
80. Madsen, J. A., Forsyth, D. W. & Detrick, R. S. *J. geophys. Res.* **89**, 9997-10015 (1984).
81. Senske, D. A. & Head, J. W. *Lunar planet. Sci.* **19**, 1063-1064 (1988).
82. Olson, P. & Nam, I. *J. geophys. Res.* **91**, 7181-7191 (1986).
83. Smrekar, S. & Phillips, R. J. *Lunar planet. Sci.* **21**, 1176-1177 (1990).
84. Herrick, D. L. & Parmentier, E. M. *Lunar planet. Sci.* **21**, 497-498 (1990).
85. Herrick, D. L. & Parmentier, E. M. *Rep. planet. Geol. Geophys. Prog.* (NASA, Washington DC, in the press).
86. Head, J. W., Vorder Bruegge, R. W. & Crumpler, L. S. *Geophys. Res. Lett.* (in the press).
87. Head, J. W., Crumpler, L. S. & Fisher, P. C. *Lunar planet. Sci.* **19**, 475-476 (1988).
88. Richards, M. A., Duncan, R. A. & Courtillot, V. E. *Science* **246**, 103-107 (1989).
89. Arkani-Hamed, J. & Toksoz, M. N. *Phys. Earth planet. Inter.* **34**, 232-252 (1984).
90. Solomon, S. C. *Lunar planet. Sci.* **21**, 1178-1179 (1990).
91. Grimm, R. E. & Solomon, S. C. *Geophys. Res. Lett.* **14**, 538-541 (1987).
92. Zuber, M. T. *J. geophys. Res.* **92**, 541-551 (1987).
93. Grimm, R. E. & Solomon, S. C. *J. geophys. Res.* **93**, 11911-11929 (1988).
94. Zuber, M. T. & Parmentier, E. M. *Icarus* **85**, 290-308 (1990).
95. Hess, P. C. & Head, J. W. *Earth Moon & Planet* (in the press).
96. Phillips, R. J. *J. geophys. Res.* **95**, 1301-1316 (1990).
97. Molnar, P. *Nature* **335**, 131-137 (1988).
98. Forsyth, D. W. & Uyeda, S. *Geophys. J.* **43**, 163-200 (1975).
99. Head, J. W. & Wilson, L. *J. geophys. Res.* **91**, 9407-9446 (1986).

ACKNOWLEDGEMENTS. We thank M. Parmentier, C. Sotin, D. Senske, D. Forsyth and T. Tullis for discussions, A. Hilliard, P. Neivert and M. E. Murphy for help with manuscript preparation, and A. T. Basilevsky, W. Kaula, J. Wood, R. Grimm, D. Senske, K. Roberts, R. Vorder Bruegge, E. Stofan, A. deCharon and J. Aubele for reviews of an earlier draft. This work was supported by NASA.

Sequential initiation of lagging and leading strand synthesis by two different polymerase complexes at the SV40 DNA replication origin

Toshiki Tsurimoto, Thomas Melendy & Bruce Stillman

Cold Spring Harbor Laboratory, PO Box 100, Cold Spring Harbor, New York 11724, USA

Enzymatic synthesis of DNA from the simian virus 40 origin of DNA replication has been reconstituted *in vitro* with eight purified components. DNA polymerase α -primase complex first initiates DNA synthesis at the replication origin and continues as the lagging strand polymerase. Subsequently, the DNA polymerase δ complex initiates replication on the leading strand template. Some prokaryotic DNA polymerase complexes can replace the eukaryotic polymerase δ complex. A model for polymerase switching during initiation of DNA replication is presented.

REPLICATION of simian virus 40 (SV40) DNA is an ideal system to study the biochemistry of eukaryotic chromosomal DNA replication. A watershed in these studies was the development of cell-free extracts from primate cells that could support replication of plasmid DNAs containing the SV40 origin of DNA replication (SV40 *ori*) in the presence of SV40 large tumour (T) antigen¹⁻⁴. Subsequent characterization of this cell-free system has led to a detailed understanding of the mechanism of DNA replication and the functions of novel replication proteins in human cells (reviewed in refs 5, 6 and 7). Perhaps the most surprising development from a mechanistic point of view was the suggestion that, unlike DNA replication in prokaryotes, two different DNA polymerases were required for eukaryotic DNA replication⁸⁻¹⁴.

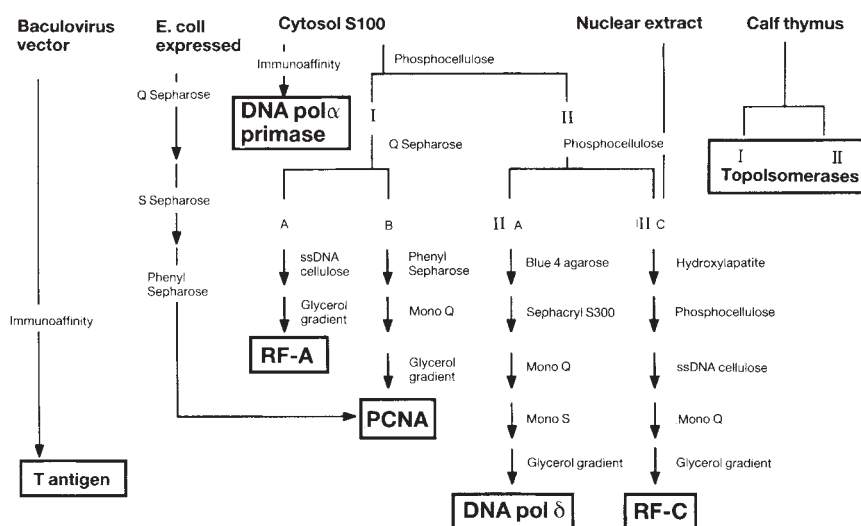
The replication reaction has a characteristic time lag of 8-10 min before the start of DNA synthesis at the SV40 *ori* and this has been defined as the presynthesis stage^{7,15-19}. This stage requires only three purified proteins: T antigen, replication factor A (RF-A, also called RP-A or eukaryotic SSB) and

topoisomerase I or II. During this period, T antigen first forms a multisubunit complex with SV40 *ori* sequences in the presence of ATP²⁰⁻²² and causes local unwinding of the *ori* DNA. In the presence of RF-A and a topoisomerase, the DNA helicase of T antigen then functions to unwind the *ori* region more extensively and form an unwound complex at the origin. This unwound complex is recognized by the DNA replication machinery, which then initiates DNA synthesis and coordinately replicates both leading and lagging strand templates.

Compared with our understanding of the presynthesis stages, relatively little is known about the subsequent initiation of both lagging and leading strand DNA synthesis at the replication origin, although several unique features of the eukaryotic replication fork have been elucidated. DNA polymerase α (pol α), which is the predominant DNA polymerase in eukaryotic cells and is tightly associated with primase activity, was thought for a long time to be the only replicative polymerase²³. When, however, the proliferating-cell nuclear antigen (PCNA) was isolated as a replication factor and further identified as a processivity factor for DNA polymerase δ (pol δ) (refs 8, 9), a role for this novel DNA polymerase was suggested. Subsequent characterization of PCNA and another DNA replication factor, RF-C, suggested that both DNA polymerases function during DNA replication^{11,12,24,25}. This model was further supported by genetic and biochemical studies of *Saccharomyces cerevisiae* DNA polymerase III, which is the pol δ equivalent in yeast²⁶⁻²⁸.

RF-C is a DNA-dependent ATPase and a primer-binding protein which also alters the activities of both pol α and pol δ ^{12,29}. The RF-C ATPase activity is stimulated by PCNA. The characterization of RF-C and PCNA suggested functional similarity to the bacteriophage T4 DNA polymerase accessory proteins encoded by the genes 44/62 and gene 45, respectively²⁹. Omission of either PCNA or RF-C from the replication reactions results in the accumulation of short nascent DNA strands that hybridize to the lagging strand template^{11,24}. This implies a functional asymmetry of DNA synthesis at the replication fork.

FIG. 1 Fractionation flowchart for the purification of human cell replication factors. Details of the purification procedures for T antigen³, RF-A (ref. 45), PCNA (ref. 8), RF-C (ref. 24), and topoisomerases I and II (ref. 18) have been published. In this report, human PCNA purified from an *E. coli* producing strain was used (K. Fein and B.S., unpublished). Human pol δ was purified by a five step procedure from fraction IIA (T.M. and B.S., manuscript submitted). Human pol α -primase complex was purified by immunoaffinity chromatography from human 293 cell S100 according to a published method^{32,33}. In addition to the human pol δ shown, DNA pol δ was also prepared from calf thymus following a published protocol⁴⁶ with slight modifications and the single-stranded DNA cellulose fraction was used. The boxed components are highly purified, essential replication factors.



Although these studies have strongly implicated a role for two DNA polymerases in eukaryotic DNA replication, this has not been directly demonstrated and a number of functions for two DNA polymerases have been proposed³⁰. We have reconstituted DNA replication from the SV40 *ori* with highly purified proteins and demonstrate directly that pol α -primase functions to initiate DNA replication at *ori* and subsequently functions as the lagging strand polymerase. In contrast, pol δ , RF-C and PCNA are required for initiation of leading strand synthesis at *ori* and function as the leading strand replicative complex. Surprisingly, the leading strand polymerase complex can be replaced by similar complexes from two bacteriophages. On the basis of these results, we propose a general model for DNA replication, involving DNA polymerase switching during initiation.

SV40 DNA replication *in vitro*

In previous experiments, SV40 DNA replication *in vitro* was reconstituted with six purified components (see Fig. 1) and one fraction that contained multiple components (Fraction IIA)¹⁸. Recently, this fraction has been further divided into two essential factors. On further fractionation, it became clear that one of these cofractionated through several chromatographic steps with a DNA polymerase activity that was similar to pol δ in that it was stimulated by PCNA with poly(dA)/oligo(dT) as a template/primer (T.M. and B.S., submitted for publication). Another component cofractionated with pol α /primase activity. Therefore, it seemed that two DNA polymerases were necessary for replication *in vitro*.

Previous studies with singly primed, single-strand DNA templates showed that RF-A, RF-C and PCNA stimulated the activities of either pol α or pol δ ^{12,25}. Characterization of the sizes of the reaction products from such experiments was consistent with the proposal that pol α synthesized the short lagging strand Okazaki fragments and that pol δ synthesized the longer leading strands¹². All of these observations implied that purified DNA polymerases α and δ were the two crucial replication factors present in Fraction IIA. Therefore, DNA replication was reconstituted *in vitro* with the purified replication factors shown boxed in Fig. 1, except that pol δ was purified either from human 293 cells (as shown) or from calf thymus tissue.

In these reconstitution experiments and in other studies on the functions of RF-C, RF-A and PCNA (T.T. and B.S., submitted for publication), it became clear that the relative amounts of the various replication factors were critical for obtaining replication intermediates that reflected the intermediates found with the crude extracts and with those found *in vivo*. Of particular importance was the amount of RF-A in the replication reaction. With subsaturating amounts of RF-A (10–15 $\mu\text{g ml}^{-1}$), synthesis of DNA on both the leading and lagging strands occurred when pol α was the only polymerase, as has been reported previously^{31,32}. With higher amounts of RF-A (>50 $\mu\text{g ml}^{-1}$), however, the synthesis of DNA on the leading strand template by pol α was minimal and a requirement for the replication factors PCNA and RF-C, as well as pol δ became apparent (see below; T.T. and B.S., submitted for publication).

A detailed analysis of the products of the reconstituted replication reaction is shown in Fig. 2. A time course of DNA synthesis revealed a time lag of approximately 8 min before the incorporation of dAMP (Fig. 2a), which corresponded to time of formation of the unwound complex^{7,18}. Thereafter, DNA synthesis had kinetics similar to reactions with crude cell extracts. The earliest reaction products hybridized preferentially to a DNA fragment (285 base pairs (bp)), that contained the *ori* sequences (Fig. 2b, 8 min), whereas all fragments from the template hybridized to reaction products from a later time point (Fig. 2b, 64 min). At intermediate time points, the pattern of hybridization indicated that DNA replication proceeded bidirectionally from *ori* in a population of molecules (data not shown). The reaction products from the time course were also purified and analysed

by gel electrophoresis in neutral (Fig. 2c) and alkaline (Fig. 2d) conditions. These results demonstrate that replication intermediates migrating between forms I and II appeared at early times and products migrating at the position of form II marker DNA first appeared at 24–32 min (Fig. 2c). A size analysis of the denatured replication products (Fig. 2d) demonstrated that at early times, short replication products were observed, but at later times, a bimodal pattern accumulated. As shown below

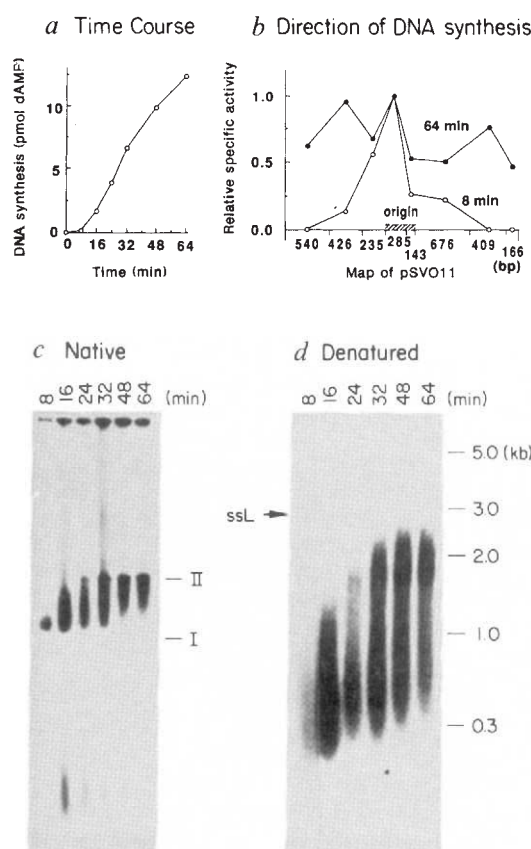


FIG. 2 Analyses of the replication products. *a*, Time course of DNA replication. The components of a complete reaction were assembled on ice and then incubated in the presence of [α -³²P]dATP at 37 °C for the indicated times. A sample of the reaction mixture was withdrawn at each time point and measurement of dAMP incorporation and product analyses were carried out. *a*, Time course of DNA synthesis measured by the incorporation of dAMP in a 25- μl reaction mixture. *b*, Location of the initiation site and direction of DNA synthesis. ³²P-labelled replication products isolated from reactions stopped after 8 min, 16 min (not shown) and 64 min were denatured and hybridized to a filter (GeneScreen plus (NEN)) containing denatured fragments of pSV011 digested with *Dde*I and *Sph*I. After the autoradiography, the bands were measured by densitometric tracing. The area under each peak was divided by the length of each fragment to obtain the specific activity, and the relative specific activity of each fragment is indicated, normalized to 1.0 for the 285-bp origin fragment. The 16-min time-point showed that the replication in a population of molecules was bidirectional from the origin (data not shown). A portion of the reaction mixture from each time point was mixed with an equal volume of Stop solution (0.2 mg ml⁻¹ of proteinase K, 2% SDS and 20 mM EDTA) and incubated at 37 °C for 1 h. The DNA sample was extracted with phenol/chloroform (1:1), precipitated with ethanol, dissolved in 10 mM Tris HCl, pH 7.4 and 1 mM EDTA. Samples of the replication products were separated in either a 0.8% native (*c*) or a 1.0% denatured (*d*) agarose gel. The same number of counts (except for the 8 min sample where one third of the counts were loaded) were loaded for gel electrophoresis. A twofold longer exposure is shown for the 8-min sample in (*d*). Native gel electrophoresis was in Tris-borate-EDTA buffer⁴⁷ and alkaline gel electrophoresis was in 30 mM NaOH and 1 mM EDTA. The numbers at the side of the autoradiograms refer to the markers. In *d*, the denatured DNA length was estimated from ³²P-labelled adenovirus DNA cut with *Hind*III and run in parallel. I, II and ssL indicate the positions of form I, form II and single-stranded linear pSV011 respectively.

(Fig. 3c), the shorter products at the 64-min time point correspond to lagging strand DNAs, whereas the longer products are leading strand products. In this minimal reconstituted system, the lagging strand products were not ligated together as the enzymes that remove the RNA primers and ligate the DNA fragments are absent³². In every other respect, however, the replication products correspond to those observed in reactions with crude cell extracts and during SV40 infection *in vivo*^{2,3,5,6}.

Proteins required for DNA replication

The complete system contains RF-A, PCNA, RF-C, immunopurified pol α -primase complex and calf thymus pol δ in addition to T antigen and topoisomerases I and II (Table 1 and Fig. 3a, b). DNA pol δ from either calf thymus or human 293 cells gave essentially the same results (Fig. 3a, b, lanes 4 and 5), demonstrating that, in contrast to a previous report¹³, there is relaxed species specificity for this polymerase, unlike the species specificity for the pol α -primase complex³³. This replication system was absolutely dependent on SV40 *ori* and T antigen (lanes 1 and 2). Omission of either RF-A or pol α -primase also eliminated DNA replication (lanes 7 and 8), confirming that both pol α -primase and RF-A are required for initiation. Under these conditions, when either pol δ , PCNA or RF-C were omitted from the reaction, DNA synthesis was reduced and the reaction products resembled those previously found when either PCNA or RF-C were depleted from the crude extract system^{11,24} (Table 1 and Fig. 3a, b, lanes 9–11). These partial replication products corresponded to the shorter products from the bimodal distribution of replication products obtained with the complete system (Fig. 3b, compare lanes 4 and 5 with 9–11). It is noteworthy that similar reaction products were observed when either pol δ , RF-C or PCNA were omitted from the reactions. Previously, we have demonstrated that pol δ , PCNA and RF-C cooperate with RF-A to synthesize long DNA strands on singly primed, single-strand M13 DNA templates and may function together as an independent replicative complex¹². In other experiments, short reaction products were observed when T antigen, RF-A, pol α -primase complex and topoisomerases I and II were the only replication proteins added to the reactions (see Fig. 4), again emphasizing that pol α and pol δ synthesize different reaction products.

Synthesis of leading and lagging strands

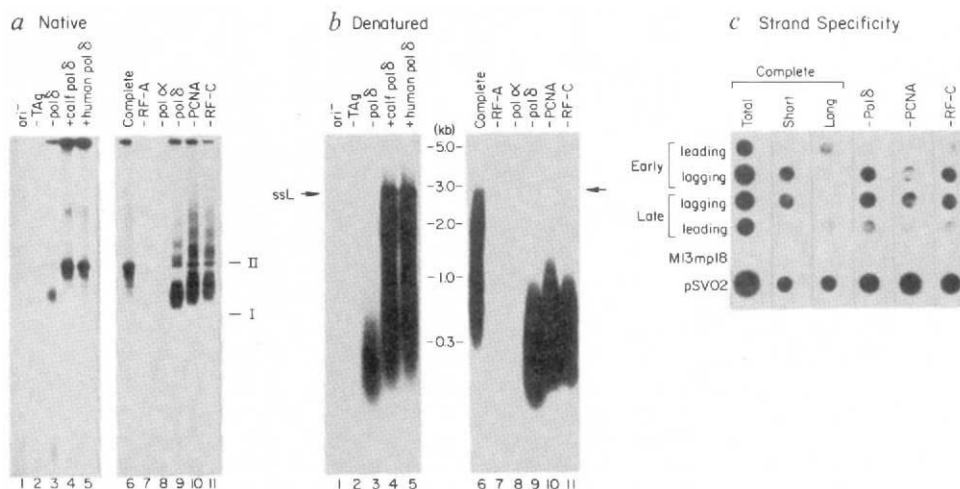
Our previous characterization of the mechanism of DNA replication^{11,12,24} and the bimodal distribution of DNA replication products synthesized by two different DNA polymerases (Figs 2 and 3) suggested that the long and short products may represent nascent leading and lagging strands respectively. This was tested by excising the ³²P-labelled long and short nascent strands from an alkaline agarose gel and hybridizing them to immobilized phage M13 DNAs containing cloned leading or lagging strand template SV40 DNA from both sides of *ori* (ref. 11 and Fig. 3c). In this experiment, to avoid cross-hybridization with the phage M13 DNA, the pSV02 plasmid DNA was used as a template¹¹. From a complete reconstituted reaction, the isolated short and long fragments preferentially hybridized with the lagging and leading strand templates, respectively, from both sides of the *ori*, whereas the total reaction products hybridized to all four strands as expected (Fig. 3c, complete).

As noted above, omission of either pol δ , RF-C or PCNA resulted in the accumulation of short replication products. With pSV02 as a template DNA, total ³²P-labelled products from reactions that lacked pol δ , RF-C or PCNA were hybridized to the M13 recombinant phage DNAs to check for strand specificity (Fig. 3c). In each case, the replication products preferentially hybridized to the lagging strand template DNA from both sides of *ori*. This confirms previous data with crude replication extracts depleted for RF-C (ref. 24) and PCNA (ref. 11), but in addition these results directly demonstrate that in the absence of pol δ , leading strands are not synthesized. Therefore, pol δ is the leading strand DNA polymerase. Furthermore, because the replication reactions were performed with purified proteins, these results also directly demonstrate that pol α is the DNA polymerase that synthesizes the lagging strands.

Substitution by phage DNA polymerase complexes

Species-specific interactions between replication proteins involved in the early stages of initiation of DNA replication have been observed. For example, species-specific interactions between T antigen and pol α -primase^{33–35} or RF-A and pol α -primase³⁶ have been reported. Therefore, initiation of lagging strand synthesis on the unwound complex requires highly

FIG. 3 Product analysis and dependence on individual replication factors. Samples from the replication reactions were analysed by a native agarose electrophoresis (a), denatured agarose gel electrophoresis (b) and by 'dot-blot' hybridization for strand specificity (c). For the gels in a and b, samples from the replication reactions described in Table 1 (experiment 1 on the left of each panel (lanes 1–5) and experiment 2 on the right of each panel (lanes 6–11)) were used. The ³²P-labelled DNA from a portion of the reaction was isolated as described in the legend to Fig. 2 and the following amounts were subjected to gel electrophoresis. For the reactions from experiment 1, the same proportion of each sample was used. For the reactions from experiment 2, the same number of counts were loaded, except for the reactions that had almost no DNA synthesis (lanes 7 and 8; –RF-A and –pol α) where the same volume as the –pol δ reaction was loaded (lane 9). For panel c, the pSV02 plasmid was replicated under the same conditions as for experiment 2 in Table 1. The products were purified, digested with *RsaI* and *DdeI*, denatured and hybridized to strand-specific M13 DNAs containing the individual strands from either side of *ori* as shown. These M13 recombinant phage have been described



previously¹¹. The isolated ³²P-labelled DNA from a complete reaction was hybridized directly (total) or was subjected to denatured gel electrophoresis and the long (1.5–4 kb) or short (0.2–0.8 kb) products were isolated and used. For the three reactions shown on the right, the products obtained in the absence of the indicated components were isolated and hybridized directly to the filter. The filter contained recombinant M13 DNAs or denatured pSV02 DNA as indicated.

To test for the specificity of protein-protein interactions during the later stages of replication, we tested an extreme case; an exchange of the leading strand DNA polymerase complex (pol δ , RF-C and PCNA) with prokaryotic DNA polymerase complexes from bacteriophages T4 and T7, as well as from *E. coli*. These well-characterized DNA polymerase complexes are required for leading strand DNA synthesis in each system and we have recently reported similarities in function between the phage T4 44/62 and 45 proteins and RF-C and PCNA respectively²⁹. As shown in Fig. 4, replication reactions containing T antigen, topoisomerases I and II, RF-A and pol α -primase synthesized short, lagging strands (lane 1), whereas the addition of pol δ , RF-C and PCNA restored complete replication (lane 2). The addition of polymerase complexes from either phage T4 (43 (DNA polymerase), 44/62 (DNA-dependent ATPase and primer-binding protein) and 45 (equivalent to PCNA)) or phage T7 (DNA polymerase coupled to *E. coli* thioredoxin) work as well as the human leading strand DNA polymerase complex, although higher levels of polymerase activity were required to obtain equivalent levels of DNA synthesis (lanes 3 and 7). These products consist of short and long DNA strands which hybridize to the lagging and leading strand template DNAs, respectively, and are therefore indistinguishable from the products with the human system. In contrast, a wide concentration range of *E. coli* pol III holoenzyme, containing all the proteins required for leading strand replication, did not support complete replication (lanes 8-10), although it was fully active on primed, single-strand DNA templates (Fig. 4 legend). Moreover, no combination of *E. coli* pol III and its accessory proteins substituted for the human pol δ complex (data not shown). Interestingly, all the T4 proteins were required for complete replication (lanes 3-6) and none of the individual T4 proteins could substitute for individual human proteins (data not shown). This is consistent with the previous demonstration that pol δ , PCNA and RF-C are functionally equivalent to the T4 43, 45 and 44/62 proteins, respectively. It also emphasizes that the proteins function as an autonomous replicative unit and suggests that the T4 proteins can cooperate with human RF-A. In this case, however, there is probably no specific interaction between the leading and lagging strand complexes, but rather, the leading strand complex follows lagging strand synthesis.

Discussion

It is clear from these results *in vitro* that two separate DNA polymerases are required for replication from the SV40 *ori*. As both DNA pol I (ypol α) and pol III (ypol δ) are essential during S phase in the yeast *S. cerevisiae*²⁶⁻²⁸, it is likely that the results presented here can be generalized to DNA replication from origins within cellular chromosomes. Furthermore, having reconstituted the replication reaction *in vitro* with purified components, we can study the biochemical 'phenotype' of reactions lacking individual components and fathom the mechanism of initiation of both the leading and lagging strand DNAs.

DNA pol α is required for initiation of DNA replication at *ori* and for lagging strand DNA synthesis. As this DNA polymerase is tightly associated with primase, the initiation of DNA replication at *ori* most probably occurs by a primase-mediated RNA priming mechanism which is consistent with the mechanism *in vivo*³⁷. Previous reports have demonstrated that pol α -primase complex from primate species (but not bovine or murine sources), will support SV40 DNA replication *in vitro*^{10,32,33} and a physical interaction between pol α -primase complex and T antigen has been suggested^{34,35}. Therefore, initiation of DNA

replication in this minimal reconstituted system probably involves specific protein-protein interactions between T antigen, RF-A and the pol α -primase complex. As T antigen is the only DNA helicase that functions during lagging strand synthesis, it most probably moves away from *ori* with the replication fork, as has been suggested previously^{38,39}. The first nascent strands synthesized at the replication origin seem to be the first Okazaki fragments for lagging strand replication, with the pol α -primase complex moving with T antigen away from the origin to become the lagging strand DNA polymerase. Most importantly, the results reported here demonstrate directly that the first nascent strands synthesized at the replication origin are synthesized by the lagging strand polymerase (pol α) and that a DNA polymerase switch must occur to initiate leading strand synthesis.

DNA pol δ is required for the synthesis of the leading strands on both sides of the *ori* and functions with the accessory proteins RF-C and PCNA. RF-A binds single-strand DNA and, in addition to acting with T antigen to form the unwound complex, stimulates pol α activity by itself and cooperates with RF-C and PCNA to stimulate pol δ activity^{24,25}. The results presented here also suggest that RF-A contributes to the specificity of leading and lagging strand synthesis by pol α and pol δ . The concentration of RF-A in the reaction is an important determinant in the integration of the leading strand polymerase complex into the replication fork and in the elimination of improper leading strand synthesis by the lagging strand polymerase complex. One subunit of RF-A is phosphorylated in a cell-cycle dependent manner in both human and yeast cells, with phosphorylation first appearing at the G₁-S phase transition⁴⁰. Although a functional difference amongst the multiple

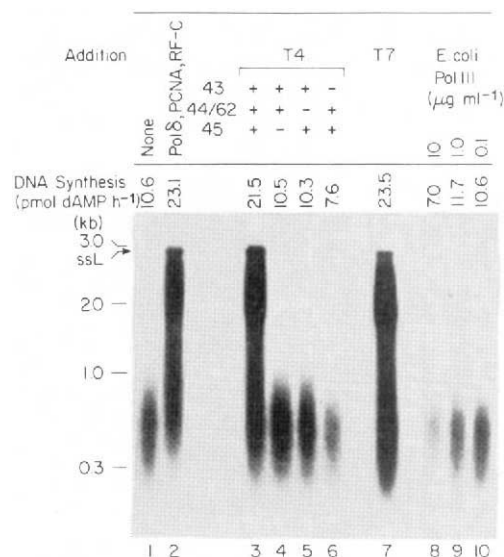


FIG. 4 Replacement of the leading strand complex with prokaryotic DNA polymerase complexes. Reactions were carried out as described in Table 1. 'None' represents a reaction with T antigen, topoisomerases I and II, RF-A and pol α -primase (lane 1). To this core reaction were added lane 2: pol δ , PCNA and RF-C; lane 3: purified T4 replication proteins 44/62, 45 and 43; lane 4: same as 3, except without 45; lane 5: same as 3, except without 44/62; lane 6: same as 3, except without 43; lane 7: T7 DNA polymerase complex; lanes 8-10: various amounts of *E. coli* pol III holoenzyme. The products from the experiment were purified and subjected to denaturing agarose gel electrophoresis. The amount of DNA synthesis for each reaction is indicated at the top of each lane. Modified T7 DNA polymerase (Sequenase II) was purchased from USB. Components added were 0.1 $\mu\text{g ml}^{-1}$ T4 gene 43 protein, 10 $\mu\text{g ml}^{-1}$ gene 45 protein, 5 $\mu\text{g ml}^{-1}$ genes 44/62 complex, 0.012 units ml^{-1} T7 DNA polymerase and various amounts of *E. coli* pol III holoenzyme. The relative DNA polymerase activities of pol δ , T4, T7 and *E. coli* pol III (at 10 $\mu\text{g ml}^{-1}$) were measured under the same reaction conditions with poly(dA)/oligo(dT) (20:1) as a template and gave ratios of 1:3:13:4 respectively.

forms of RF-A has not been demonstrated, it is possible that the phosphorylation of RF-A affects a number of these replicative functions.

Both DNA polymerases used in this study are highly purified, but may not be homogeneous. But the replacement of RF-C, PCNA and pol δ with the phage DNA polymerase complexes and the similar dependence on the individual phage T4 or human proteins eliminates the possibility that the human proteins are contaminated with other essential replication factors. This approach, which is analogous to interspecies genetic complementation *in vivo*, enables us to make definitive statements about the need for the human proteins during DNA replication *in vivo*, as the phage T4 43, 44/62 and 45 proteins are clearly required for phage DNA replication (reviewed in ref. 41). Recently, the 44/62 and 45 proteins have also been shown to stimulate transcription of the phage T4 late genes *in vitro*, implicating these proteins in the DNA replication dependent switch to late gene expression during phage infection⁴². As the RF-C and PCNA proteins are functionally similar to the T4 proteins, there remains the possibility that they may coordinate DNA replication-dependent changes in cellular and viral transcription.

It is striking that some bacteriophage DNA polymerase complexes can function as the leading strand polymerase complex following initiation at *ori* by pol α -primase. Although there are functional and structural similarities between the T4 and human proteins^{29,43}, this is not the only reason that they can substitute, as the unrelated phage T7 leading strand polymerase complex can also work. Not all DNA polymerase complexes could substitute, however, as the *E. coli* pol III complex did not work and obviously, pol α did not continue as the leading strand polymerase. These results can be explained by the fact that pol δ and the phage polymerase complexes could synthesize DNA on primed single-strand templates in the presence of high concentrations of RF-A, whereas pol α and the *E. coli* pol III were inactive under these conditions (T.T., T.M. and B.S., unpub-

lished observations). This polymerase specificity in the presence of high concentrations of RF-A probably reflects part of the mechanism of DNA polymerase switching from pol α to pol δ .

PCNA and RF-C function as an additional control mechanism for integration of the leading strand polymerase complex into the replication reaction (T.T. and B.S., submitted for publication). RF-C can stimulate DNA synthesis by pol α (ref. 12) and thus may function as a lagging strand polymerase accessory factor; however, when PCNA and RF-C form a complex on primed single-strand DNA in the presence of ATP, it functions as a primer recognition protein and blocks pol α from priming on the 3' ends of Okazaki fragments (ref. 29 and T.T. and B.S., submitted for publication). This places the RF-C-PCNA complex in a convenient location for promoting initiation of leading strand synthesis by pol δ . Indeed we have shown that RF-C and PCNA cooperate with RF-A to stimulate pol δ activity on primed DNA templates in an ATP-dependent manner¹². Therefore, RF-C participates in a molecular mechanism to switch from the initiation of DNA synthesis at *ori* by pol α to the initiation of leading strand synthesis by pol δ . The combination of a high concentration of RF-A and a leading-strand specific primer-recognition complex ensures that pol δ can be integrated efficiently into the replication fork as a leading strand polymerase complex and the pol α function is limited to the lagging strand template. These results show that initiation of DNA replication at *ori* involves the sequential assembly of several multiprotein complexes.

A model for bidirectional DNA replication

The results discussed above suggest a model for the initiation of bidirectional DNA replication on both leading and lagging strand templates (Fig. 5). Once the *ori* sequences are unwound by the combined action of T antigen and RF-A, pol α -primase complex binds to the newly exposed single-stranded region, perhaps mediated by protein-protein interactions with T antigen and/or RF-A, and synthesizes short oligoribonucleotides that

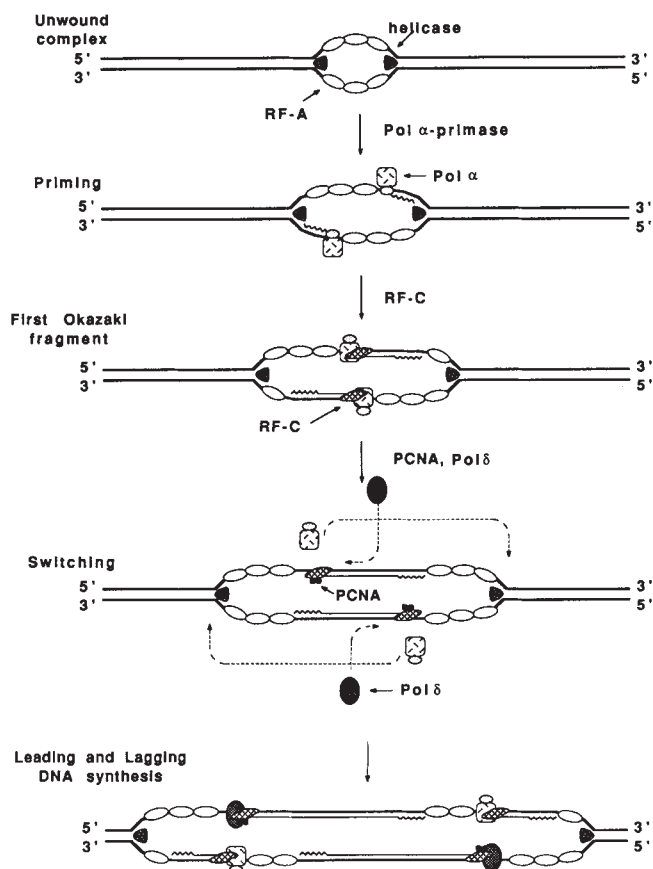


FIG. 5 Model for initiating leading and lagging strand synthesis with two DNA polymerases. The first line of the figure shows the unwound complex stage and then five subsequent stages: primer RNA synthesis with primase (priming), first Okazaki fragment synthesis with pol α , switching of DNA polymerases at the 3' end of this first Okazaki fragment, and finally, leading and lagging strand synthesis by pol δ and pol α are indicated (see text for details). Factors involved are DNA helicase (T antigen in the case for SV40), RF-A, pol α -primase, RF-C, PCNA and pol δ . The factors are introduced according to the order of entry into the reaction. The role of the topoisomerases are not indicated. Thick and thin lines are template and newly synthesized DNAs respectively. Wavy lines are synthesized primer RNAs and the dashed lines indicate the introduction of pol δ and the movement of pol α -primase complex along the lagging strand template without dissociation from the replication fork.

TABLE 1 Requirements for SV40 DNA replication *in vitro*

Component omitted or added	DNA synthesis (pmol dAMP h ⁻¹)
Experiment 1	
Complete	28.8
-pSV011, +pSV011ori-	0.4
-Tag	0.0
-calf thymus pol δ	5.4
-calf thymus pol δ , +human pol δ	18.5
Experiment 2	
Complete	20.0
-RF-A	0.1
-pol α	0.1
-pol δ	3.7
-PCNA	8.1
-RF-C	6.1

SV40 DNA replication with eight purified replication factors. The reaction was carried out under standard replication conditions⁵ in the presence of 6 $\mu\text{g ml}^{-1}$ of plasmid pSV011 (3.1 kilobases (kb)) or its *ori*-derivative pSV011ori⁻, 80 $\mu\text{g ml}^{-1}$ T antigen, 56 $\mu\text{g ml}^{-1}$ RF-A, 4 $\mu\text{g ml}^{-1}$ calf thymus topoisomerase I, 1.8 $\mu\text{g ml}^{-1}$ calf thymus topoisomerase II, 24 $\mu\text{g ml}^{-1}$ pol α -primase, 8 $\mu\text{g ml}^{-1}$ PCNA, 2.4 $\mu\text{g ml}^{-1}$ RF-C and 16 $\mu\text{g ml}^{-1}$ calf thymus pol δ (5.5 $\times 10^3$ units mg^{-1} in the presence of PCNA) at 37 °C for 1 h. After incubation, the dAMP incorporated into newly synthesized DNA was measured by spotting a sample onto DEAE paper (DE81, Whatman) followed by three successive washes with 0.5 M of Na₂HPO₄, then once each with distilled H₂O and ethanol, followed by liquid scintillation counting. DNA synthesis is indicated as pmol of dAMP incorporated in a 25 μl reaction. Individual components were omitted or added as indicated. The plasmid pSV011ori⁻ is similar to pSV011, except for a small deletion in the origin region. The human pol δ added is a highly purified MonoS fraction (170 $\mu\text{g ml}^{-1}$, T.M. and B.S., manuscript submitted).

prime the first Okazaki fragment at *ori*. Pol α then synthesizes the first nascent DNA at the origin. Under some conditions, RF-C is a stimulatory factor for pol α and can increase the production of Okazaki fragments when added to the lagging strand polymerase system (data not shown); therefore pol α and RF-C probably cooperate to form an active lagging strand polymerase complex¹². After the synthesis of the first Okazaki fragment at *ori*, PCNA interacts with RF-C at the 3' end of the nascent strand and in an ATP-dependent manner forms a tight, temporary complex called the primer recognition complex. As a consequence, pol α is unable to prime on the 3' end, but pol δ efficiently recognizes the PCNA/RF-C complex and initiates

leading strand DNA replication. The dissociated pol α -primase complex cycles to the next priming site on the lagging strand template and continues in this fashion to copy the lagging strand template DNA discontinuously. If this mode of DNA polymerase switching occurred on both parental strands at *ori*, then lagging and leading strand polymerase complexes would form on both sides of *ori* and coordinated synthesis of leading and lagging strands would ensue. With the repertoire of DNA replication factors described in this report, the Okazaki fragments would not be matured by removal of the RNA primers and many gaps would remain between newly synthesized lagging strand DNA fragments. We imagine, however, that under normal circumstances the lagging strand polymerase complex would contain additional factors to fill in the gaps. In addition, the reconstituted replication reaction described in this report contains the rudimentary protein components and it is most probable that many other proteins function during all stages of DNA replication *in vivo*.

This model could also apply to the initiation of bidirectional DNA replication at prokaryotic DNA replication origins such as *oriC* and the phage λ origin, but in these systems, a single type of DNA polymerase (*E. coli* pol III) would function as a dimer in association with accessory proteins that are asymmetrically situated on the leading and lagging strand templates (reviewed in ref. 44). Uniquely in eukaryotes, the role of the leading and lagging strand polymerases has been assigned to different polypeptides. What is the advantage of this functional divergence? The two different polymerases could have evolved from an ancestral form into functionally specialized DNA polymerases for leading and lagging strand synthesis, perhaps to cope with chromatin, rather than DNA replication. Indeed, pol α and pol δ are structurally related to each other and to the phage T4 DNA polymerase, suggesting that they derive from a common ancestor (reviewed in ref. 43). Another possibility is that the existence of two distinct replicative complexes provides an opportunity for regulation of the assembly of multiple protein complexes during initiation of DNA replication. For example, unlike prokaryotic replicons, eukaryotic chromosomes contain multiple origins and DNA replication from these origins is temporally regulated throughout the S phase of the cell cycle. It is not known whether this regulation occurs at the *ori* recognition step, at the *ori* unwinding stage or at a later stage of initiation. The existence of multiple stages of initiation requiring assembly of a number of multiprotein complexes at the eukaryotic replication origin offers various regulatory possibilities. □

Received 1 May; accepted 25 June 1990.

- Li, J. J. & Kelly, T. J. *Proc. natn. Acad. Sci. U.S.A.* **81**, 6973-6977 (1984).
- Li, J. J. & Kelly, T. J. *Molec. cell. Biol.* **5**, 1238-1246 (1985).
- Stillman, B. W. & Gluzman, Y. *Molec. cell. Biol.* **5**, 2051-2060 (1985).
- Wobbe, C. R., Dean, F., Weissbach, L. & Hurwitz, J. *Proc. natn. Acad. Sci. U.S.A.* **82**, 5710-5714 (1985).
- Stillman, B. A. *Rev. Cell Biol.* **5**, 197-245 (1989).
- Challberg, M. D. & Kelly, T. J. *Rev. Biochem.* **58**, 671-717 (1989).
- Borowiec, J. A., Dean, F. B., Bullock, P. A. & Hurwitz, J. *Cell* **60**, 181-184 (1990).
- Prelich, G., Kostura, M., Marshak, D. R., Mathews, M. B. & Stillman, B. *Nature* **326**, 471-475 (1987).
- Prelich, G. *et al. Nature* **326**, 517-520 (1987).
- Wold, M. S., Weinberg, D. H., Virshup, D. M., Li, J. J. & Kelly, T. J. *J. biol. Chem.* **264**, 2801-2809 (1989).
- Prelich, G. & Stillman, B. *Cell* **53**, 117-126 (1988).
- Tsurimoto, T. & Stillman, B. *EMBO J.* **8**, 3883-3889 (1989).
- Weinberg, D. H. & Kelly, T. J. *Proc. natn. Acad. Sci. U.S.A.* **86**, 9742-9746 (1989).
- Lee, S.-H., Eki, T. & Hurwitz, J. *Proc. natn. Acad. Sci. U.S.A.* **86**, 7361-7365 (1989).
- Dean, F. B. *et al. Proc. natn. Acad. Sci. U.S.A.* **84**, 16-20 (1987).
- Wold, M. S., Li, J. J. & Kelly, T. J. *Proc. natn. Acad. Sci. U.S.A.* **84**, 3643-3647 (1987).
- Borowiec, J. A. & Hurwitz, J. *EMBO J.* **7**, 3149-3158 (1988).
- Tsurimoto, T., Fairman, M. P. & Stillman, B. *Molec. cell. Biol.* **9**, 3839-3849 (1989).
- Roberts, J. M. *Proc. natn. Acad. Sci. U.S.A.* **86**, 3939-3943 (1989).
- Deb, S. P. & Tegtmeyer, P. *J. Virol.* **61**, 3649-3654 (1987).
- Dean, F. B., Dodson, M., Echols, H. & Hurwitz, J. *Proc. natn. Acad. Sci. U.S.A.* **84**, 8981-8985 (1987).
- Mastrangelo, I. A. *et al. Nature* **338**, 658-662 (1989).
- Fry, L. A. & Loeb, L. A. *Animal Cell DNA Polymerases* (CRC, Boca Raton, FL, 1986).
- Tsurimoto, T. & Stillman, B. *Molec. cell. Biol.* **9**, 609-619 (1989).
- Kenny, M. K., Lee, S.-H. & Hurwitz, J. *Proc. natn. Acad. Sci. U.S.A.* **86**, 9757-9761 (1989).
- Bauer, G. A., Heller, H. M. & Burgers, P. M. J. *J. biol. Chem.* **263**, 917-924 (1988).
- Boulet, A., Simon, M., Faye, G., Bauer, G. A. & Burgers, P. M. J. *EMBO J.* **8**, 1849-1854 (1989).

- Sitney, K. C., Budd, M. E. & Campbell, J. L. *Cell* **56**, 599-605 (1989).
- Tsurimoto, T. & Stillman, B. *Proc. natn. Acad. Sci. U.S.A.* **87**, 1023-1027 (1990).
- Blow, J. J. *Trends Genet.* **5**, 134-136 (1989).
- Wobbe, C. R. *et al. Proc. natn. Acad. Sci. U.S.A.* **84**, 1834-1838 (1987).
- Ishimi, Y., Claude, A., Bullock, P. & Hurwitz, J. *J. biol. Chem.* **263**, 19723-19733 (1988).
- Murakami, Y., Wobbe, C. R., Weissbach, L., Dean, F. B. & Hurwitz, J. *Proc. natn. Acad. Sci. U.S.A.* **83**, 2869-2873 (1986).
- Smale, S. T. & Tjian, R. *Molec. cell. Biol.* **6**, 4077-4087 (1986).
- Gannon, J. V. & Lane, D. P. *Nature* **329**, 456-458 (1987).
- Brill, S. J. & Stillman, B. *Nature* **342**, 92-95 (1989).
- Hay, R. T. & DePamphilis, M. L. *Cell* **28**, 767-779 (1982).
- Wiekowski, M., Droge, P. & Stahl, H. J. *J. Virol.* **61**, 411-418 (1987).
- Dodson, M., Dean, F. B., Bullock, P., Echols, H. & Hurwitz, J. *Science* **238**, 964-967 (1987).
- Din, S., Brill, S. J., Fairman, M. P. & Stillman, B. *Genes Dev.* **4**, 968-977 (1990).
- Cha, T.-A. & Alberts, B. M. in *Eukaryotic DNA Replication*. *Cancer Cells* **6**, 1-10 (Cold Spring Harbor Laboratory, New York, 1988).
- Herendeen, D. R., Kassavetis, G. A., Barry, J., Alberts, B. M. & Geiduschek, E. P. *Science* **245**, 952-958 (1989).
- Wang, T. S.-F., Wong, S. W. & Korn, D. *FASEB J.* **3**, 14-21 (1989).
- McHenry, C. S. A. *Rev. Biochem.* **57**, 519-550 (1988).
- Fairman, M. P. & Stillman, B. *EMBO J.* **7**, 1211-1218 (1988).
- Lee, M. Y. W. T., Tan, C.-K., Downey, K. M. & So, A. G. *Biochemistry* **23**, 1906-1913 (1984).
- Maniatis, T., Fritsch, E. F. & Sambrook, J. *Molecular Cloning. A Laboratory Manual* (Cold Spring Harbor Laboratory, New York, 1982).

ACKNOWLEDGEMENTS. We thank J. Diffley and W. Herr for critical reading of this manuscript and J. Barry and B. Alberts (University of California, San Francisco) and M. O'Donnell (Cornell University Medical School) for generous gifts of phage and *E. coli* DNA replication proteins. We also thank J. Duffy for the artwork. T.M. was supported by a fellowship from the Damon Runyon-Walter Winchell Cancer Research Fund and the research was supported by the NIH.

Identification of a ribosome receptor in the rough endoplasmic reticulum

Adam J. Savitz & David I. Meyer*

Department of Biological Chemistry and the Molecular Biology Institute, UCLA School of Medicine, Los Angeles, California 90024, USA

Attachment of ribosomes to the membrane of the endoplasmic reticulum is one of the crucial first steps in the transport and secretion of intracellular proteins in mammalian cells. The process is mediated by an integral membrane protein of relative molecular mass 180,000 (M_r 180K), having a large (at least 160K) cytosolic domain that, when proteolytically detached from the membrane, can competitively inhibit the binding of ribosomes to intact membranes. Isolation of this domain has led to the identification, purification and characterization of the intact ribosome receptor, as well as its functional reconstitution into lipid vesicles.

PROTEINS that are intracellularly transported to their final destinations by way of the secretory pathway enter this pathway at the membrane of the rough endoplasmic reticulum (ER)¹. Nascent secreted proteins, for example, are recognized, targeted to, and translocated across the membrane of the rough ER, often before translation has ended². They are recognized when the nascent polypeptide bearing the signal sequence is bound by the signal-recognition particle (SRP)^{3,4}, a cytosolic ribonucleoprotein complex⁵. Targeting occurs through the docking of the SRP-translation complex to a rough ER-specific SRP receptor-the docking protein^{6,7}. One of the next steps in this process is the attachment of the ribosome to the ER membrane. It is therefore not surprising that models of protein translocation across ER membranes consistently postulate the presence of a cellular factor, or membrane-bound receptor, that mediates this ribosome binding^{2,8-10}.

Earlier studies on the interaction of ribosomes with the membrane of the rough ER suggested that this process was mediated only in part by the nascent polypeptide chain^{11,12}. Using a binding assay comprising labelled ribosomes and stripped (ribosome-depleted) rough microsomes, Borgese *et al.* presented data in support of the concept that ribosome-membrane association involves a proteinaceous receptor as well¹³.

To approach the isolation of a ribosome receptor, we reasoned that such a molecule would be an integral membrane protein, present only in rough ER membranes, having a large cytosolic domain that mediates the binding of ribosomes. Moreover, it seemed logical that if we could selectively cleave off the bulk of the cytosolic domain intact, it should competitively block the ability of the native receptor, in intact membranes, to rebinding ribosomes. Once isolated, it should be possible by immunochemical means to determine the integral membrane protein from which the proteolytic fragment derives. When purified and incorporated into artificial lipid vesicles, the intact receptor should be able to bind ribosomes with kinetics similar to those observed in intact ER vesicles.

Inhibition of binding by a receptor fragment

The assay that we have chosen as the basis for the purification of ribosome receptor has been described previously¹⁴. Briefly,

ribosomes were removed (stripped) from canine pancreatic microsomes by a combination of puromycin, nuclease, and salt at a high concentration. [³H]uridine-labelled ribosomes, derived from HeLa cells, were allowed to bind to the stripped microsomes; samples were mixed with concentrated sucrose and overlaid with a sucrose gradient. Centrifugation resulted in the flotation of the membranes along with any bound ribosomes. Unbound ribosomes remained at the bottom of the centrifuge tube. Levels of ribosome binding to membranes were determined by scintillation counting of floated and unfloated fractions.

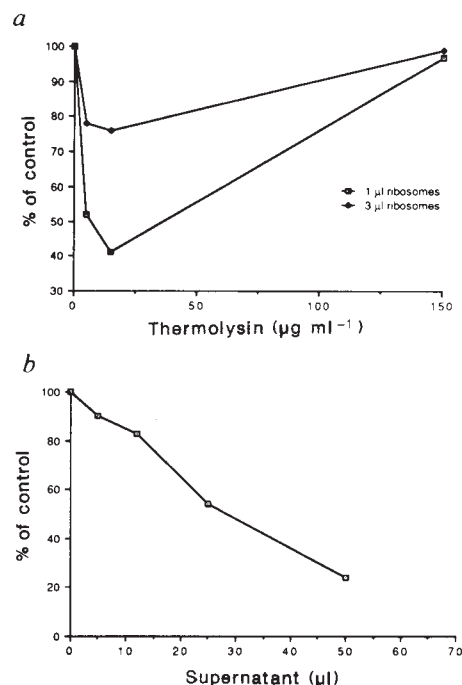


FIG. 1 Generation of inhibitory activity by thermolysin digestion of microsomal membranes. Dog pancreas microsomes that had been stripped of ribosomes (RM_{sn}) were treated with varying concentrations of thermolysin. Subsequent centrifugation yielded supernatants that were then tested for their ability to inhibit the binding of radiolabelled ribosomes to intact RM_{sn} . *a*, Inhibitory effect on ribosome binding of supernatants derived by proteolysis using increasing concentrations of thermolysin. Open symbols represent data from experiments where the concentration of ribosomes in the assay was threefold lower than in those depicted by closed symbols. *b*, The effect of increasing amounts of thermolysin supernatant on the binding of ribosomes to untreated RM_{sn} . The supernatant used was derived from RM_{sn} treated with 15 $\mu\text{g ml}^{-1}$ thermolysin.

METHODS. Labelling of HeLa cell ribosomes, preparation of dog pancreas RM_{sn} , and *in vitro* ribosome binding were carried out essentially as described by Hortsch *et al.*¹⁴, except that ribosomes and RM_{sn} were incubated at 0 °C for 10 min. Proteolysis with thermolysin (Sigma) at concentrations 5–150 $\mu\text{g ml}^{-1}$ was performed at 0 °C for 30 min and stopped with an excess of EGTA. Supernatants containing proteolytic fragments were obtained from the upper phase of a step gradient when RM_{sn} were centrifuged through a cushion of 0.5 M sucrose at 100,000*g* for 30 min. The ability of 40 μl of a given supernatant to inhibit ribosome binding, was determined by its inclusion (preincubated with labelled ribosomes for 10 min at 0 °C) in the standard (90 μl) binding assay. Per cent of control is based on the amount of ribosomes bound to the RM_{sn} when the supernatant from a mock digest (no thermolysin) was included in the binding assay.

* To whom correspondence should be addressed.

As our working model predicted that a membrane-bound ribosome receptor must have a considerable proportion of its mass on the cytosolic face of the ER membrane, we tested a variety of proteolytic enzymes for their ability to generate a hydrolysate that contained an activity (proteolytic fragments) that was inhibitory in our binding assay. Trypsin, chymotrypsin, papain, bromelain, elastase, *Staphylococcus aureus* V8 and thermolysin were all used at varying concentrations ($1\text{--}500\text{ }\mu\text{g ml}^{-1}$) to treat stripped rough microsomes. After protease inactivation, and removal of membranes by centrifugation, supernatants were tested for their ability to inhibit ribosome binding to unproteolysed, stripped membranes. Only thermolysin, at low concentrations, generated supernatants that inhibited ribosome binding to intact membranes (Fig. 1). A narrow window of proteolysis ($5\text{--}20\text{ }\mu\text{g ml}^{-1}$ thermolysin) was observed in which a fraction that inhibited ribosome binding could be obtained (Fig. 1a). The boundaries of the window were represented by the minimum concentration of thermolysin needed to generate sufficient fragments for inhibition in the binding assay, and the maximum concentration of thermolysin that could be tolerated without leading to the degradation of these fragments (data not shown). A direct relationship was observed between the amount of proteolytic supernatant and the concentration of ribosomes used in the inhibition assay; the greater the amount of proteolytic supernatant used in the assay, the greater the amount of inhibition of binding (Fig. 1b).

The next step was to purify the inhibitory factor. Consistent with the assumption that the inhibitory activity of the proteolytic digests is mediated by a fragment of the receptor being able to bind to ribosomes, we used ribosome affinity chromatography in the initial purification. Very little of the total material found in the thermolysin digests (Fig. 2a, lane 1) could bind to a column of pancreatic ribosomes (Fig. 2a). On the basis of silver-stained gels, most of the material specifically bound by the ribosome column, and eluted with 200 mM KCl, had an M_r of 160K (Fig. 2a, lane 4). The material bound and eluted from the ribosome column was enriched about 25-fold in inhibitory activity compared with the thermolysin hydrolysate (starting material) (Fig. 2b). The inhibitory activity of the eluate, as well as the 160K polypeptide, was destroyed by $3\text{--}5\text{ }\mu\text{g ml}^{-1}$ of elas-

tase. Under these conditions the other four prominent bands seen on the profile of the eluate remained intact (data not shown). From this we concluded that the inhibitory activity is a function of the 160K fragment.

Identification of the receptor

Identification of the integral membrane protein, of which the 160K fragment represents the cytosolic domain, was established by producing an antibody against the fragment. The anti-160K reactive components of the antiserum should allow detection of the protein from which the thermolysin fragment was derived, either by immunoprecipitation or immunoblotting of proteins derived from intact microsomes. Previous studies have shown that profiles of the integral membrane proteins of pancreatic rough microsomes contain only one major species that is protease-sensitive and larger than 160K¹⁴. Consistent with this was the result of immunoblotting, in which the anti-160K antibody reacted with the principal 180K rough ER-specific, carbonate-resistant (pH 11) polypeptide (Fig. 3a, lane 2).

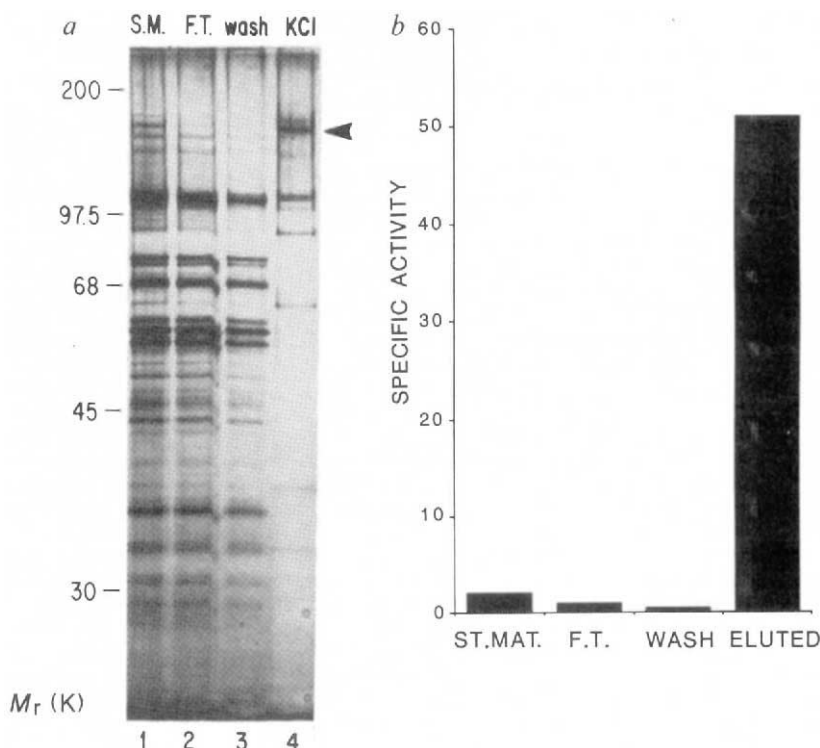
The 180K integral membrane protein has previously been shown by biochemical criteria to be a component of the rough microsomal fraction¹⁴. To verify its existence in the rough ER we used a morphological assay, indirect immunofluorescence microscopy. Permeabilized Madin-Darby canine kidney (MDCK) cells were labelled with affinity-purified anti-160K antibodies. A typical ER staining pattern was observed (Fig. 3b, c), which included the nuclear envelope, an established location of bound ribosomes. Identical patterns have been observed in this cell line by using antibodies against docking protein (SRP receptor)¹⁵. This is consistent with the presence of the antigen in the correct organelle. Unfortunately, the anti-160K antiserum does not seem to combine with an epitope that results in the inhibition of ribosome binding to intact microsomes (data not shown).

Receptor purification and ribosome binding

Several approaches could have been taken to establish that the 180K protein functions as a ribosome receptor. The best proof would be to incorporate pure 180K protein into artificial lipid vesicles and demonstrate that ribosome binding reconstituted

FIG. 2 Purification of the inhibitory protein fragment by ribosome-affinity chromatography. *a*, Protein profiles of fractions resulting from the purification of the factor inhibiting ribosome binding visualized by silver-staining. Lane 1, starting material (st. mat.), that is, the supernatant from a thermolysin digestion of RM_{sn} ; lanes 2 and 3, column flow-through (F.T.) and wash fractions respectively; lane 4, fraction eluted with 200 mM KCl. *b*, Specific inhibitory activity for ribosome binding of fractions shown in *a*. Units of specific activity of inhibition are based on the decrease in the number of ribosomes bound per mg protein in each fraction.

METHODS. Proteolysis of the RM_{sn} and the inhibition of ribosome binding were carried out as detailed in Fig. 1. The ribosome column was made as follows: canine pancreatic ribosomes isolated by the method of Blobel and Dobberstein³⁵, were resuspended in HSB (500 mM KCl, 50 mM Tris-HCl buffer, pH 7.5, 5 mM MgCl_2) and centrifuged through a 1.8 M sucrose in HSB cushion at $150,000g$ for 18 h. The pellet was resuspended in LSB (25 mM KCl, 50 mM Tris-HCl, pH 7.5, 5 mM MgCl_2) and coupled to Affigel 10 (BioRad) for 6 h at 4°C (ref. 36). The reaction was terminated by the addition of 1/10 volume of 1 M ethanolamine for 1 h at 4°C . Before use, the matrix was washed with five volumes of HSB and equilibrated in 12.5 mM KCl in TMG (50 mM Tris-HCl, pH 7.5, 5 mM MgCl_2 , 10% glycerol). The column was run with gravity flow, washed with five volumes of 25 mM KCl in TMG, and eluted with 200 mM KCl in TMG. The fractions were analysed on a 10% acrylamide SDS gel and visualized by the silver stain method of Ansorge³⁷. Protein concentrations of the fractions was determined by using Bradford reagent (BioRad)³⁸.



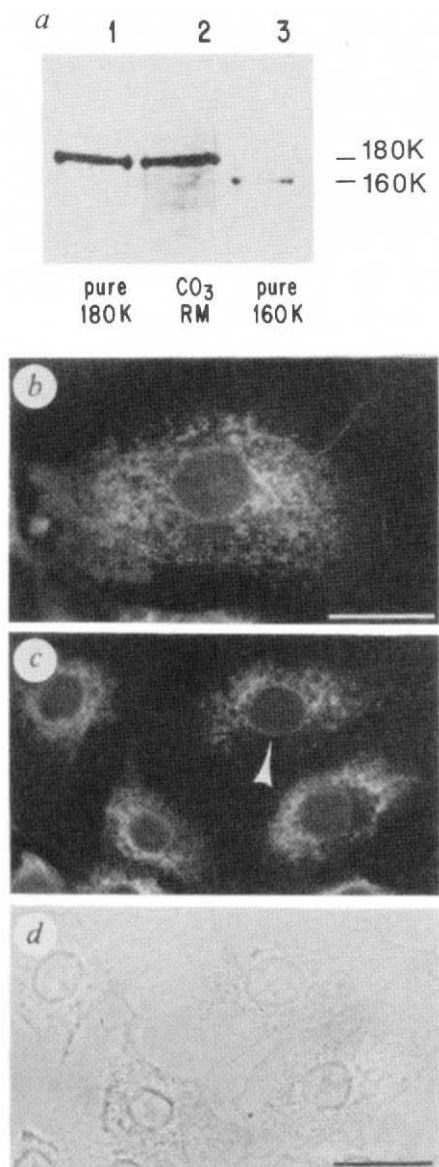


FIG. 3 Identification of the intact ribosome receptor and its localization to the endoplasmic reticulum. *a*, Immunoblot decorated with an antibody against the 160K protein fragment. Lane 1, purified intact receptor (see Fig. 4); lane 2, carbonate-washed pancreatic microsomes (RM); lane 3, thermolysin fragment eluted from ribosome-affinity column. *b*, *c*, Madin-Darby canine kidney (MDCK) cells visualized by indirect immunofluorescence using affinity-purified rabbit anti-160K ribosome receptor fragment antibody, and goat anti-rabbit rhodamine-conjugated secondary antibody. Arrowhead points out staining of the nuclear envelope, a typical site of bound ribosomes. *d*, Phase-contrast microscopy of same field of cells as shown in *c*. Scale bars 10 μ m.

METHODS. Immunoblotting: Carbonate (pH 11)-washed pancreatic microsomes were prepared by the method of Fujiki *et al.*³⁹ as modified by Hortsch *et al.*⁴⁰; purified 160K fragment, and 180K intact receptor were prepared as described in the legends to Figs 2 and 4, respectively. The samples were run on a 10% acrylamide-SDS gel, electrophoretically transferred to nitrocellulose, and decorated with rabbit anti-160K antiserum. Visualization was achieved by application of an alkaline phosphatase-conjugated goat anti-rabbit IgG secondary antibody. Immunofluorescence: MDCK cells were grown on glass coverslips for 2 days, and fixed in paraformaldehyde⁴¹. Pemeabilization was accomplished with methanol at -20°C , and cells were labelled with antibodies as described by Fukui *et al.*⁴². Antibodies used for immunofluorescence were affinity-purified by binding to samples of 160K or 180K receptor, that had been electroblotted onto nitrocellulose, and elution with 0.2 M HCl-glycine, pH 2.2.

in this way exhibits the properties of intact microsomal membranes. To accomplish this, a protocol was devised for the purification of large amounts of the intact, putative receptor (results are shown in Fig. 4). On the basis of the fact that the 160K fragment/ribosome interaction is stable up to about 200 mM KCl, we devised a three-step detergent extraction procedure in which increasing amounts of salt were used at each stage. In this way, proteins associated with ribosomes maintained this interaction during the first two fractionation steps, and were released at the third. The ribosome-associated proteins were then subjected to anion-exchange chromatography which allowed the purification of the 180K species to homogeneity (see Fig. 4 legend). Immunoblotting confirmed its

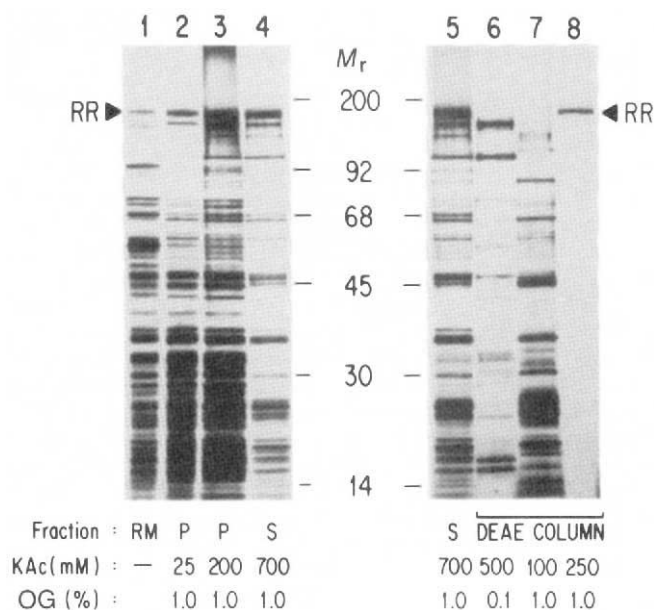


FIG. 4 Purification of the intact ribosome receptor. Top, protein profiles of samples obtained during the purification of the intact ribosome receptor as visualized by silver-staining. Lane 1, pancreatic microsomes (RM); lane 2, insoluble material (P) obtained by treatment of pancreatic microsomes with 0.5% Nikkol (not octyl-glucoside as shown) at low salt (25 mM potassium acetate (KAc)); lane 3, insoluble material derived from treatment of material shown in lane 2 with 1% octyl-1- β -D-glucopyranoside (OG) at medium salt (200 mM CH_3COOK); lane 4, soluble proteins (S) derived from 1% OG-high salt (700 mM CH_3COOK) extraction of the material shown in lane 3. Lane 5, starting material for DEAE-Sephacel chromatography, that is, the fraction shown in lane 4 diluted sevenfold; lane 6, material eluted from DEAE-Sephacel using low detergent (0.1% OG) and high salt (500 mM CH_3COOK); lane 7, proteins eluted from DEAE with 100 mM KOAc in 1% OG (note presence of ribophorin I at 68K and ribophorin II as the doublet just below); lane 8, material eluted from DEAE at 250 mM CH_3COOK in 1% OG. RR, ribosome receptor. *M_r*, calibration is shown in thousands.

METHODS. HSB-washed pancreatic microsomes were diluted with 25 mM CH_3COOK in TMG. Nikkol was added to a final concentration of 0.5%, and the suspension was centrifuged at 100,000g for 1 h at 4°C . The pellet was resuspended in 200 mM CH_3COOK , 1% OG in TMG, and centrifuged over a cushion of 1.8 M sucrose, 200 mM CH_3COOK , 1% OG in TMG in a Beckman 80Ti rotor at 175,000g for 1.5 h at 4°C . The pellet was resuspended in 700 mM CH_3COOK , 1% OG in TMG and centrifuged over a cushion of 1.8 M sucrose, 700 mM CH_3COOK , 1% OG, in TMG in a Beckman TLA 100.2 rotor at 288,000g for 20 min at 4°C . The supernatant was diluted to a final concentration of 100 mM CH_3COOK , 0.14% OG, in TMG and mixed with equilibrated DEAE-Sephacel CL-6B (Pharmacia). After pouring a column and collecting the flow-through, the column was eluted in a step-wise fashion first with five volumes of 500 mM CH_3COOK , 0.1% OG, in TMG followed by equilibration of the salt concentration to 100 mM CH_3COOK . This was followed by elution with five volumes of 100 mM CH_3COOK , 1% OG, in TMG and lastly, a gradient of 100–400 mM CH_3COOK (six column volumes), 1% OG in TMG. Fractions were analysed on SDS (5–15% acrylamide) gels there were silver-stained as described in Fig. 2.

relationship to the inhibitory thermolysin fragment and the main carbonate-insensitive, rough ER-specific antigen (Fig. 3a).

Reconstitution of the binding activity was accomplished in large unilamellar phospholipid vesicles. The 180K protein was assembled into liposomes made from 90% phosphatidylserine/10% phosphatidylcholine¹⁶. Significant amounts of 180K polypeptide were incorporated ($80 \text{ ng } \mu\text{l}^{-1}$), and ribosome binding observed. The ability of the large vesicles to bind ribosomes under various conditions is shown in Fig. 5a. In the standard assay, lipid-only liposomes or 180K-containing liposomes that had been treated with protease were capable of binding ribosomes to levels only marginally above background (Fig. 5a, compare columns 2 and 5 with column 1). When a fraction of other proteins (depicted in Fig. 4, lane 7) were incorporated into liposomes and tested, there was only a two- to threefold increase in ribosome-binding over background (Fig. 5a, column 3). This was the case, despite the fact that many of the proteins incorporated were transmembrane proteins with cytosolic domains of various sizes including ribophorins I and II (determined by immunoblotting, data not shown). By contrast, incorporation of the homogeneous 180K protein (from the purification shown in Fig. 4, lane 8) resulted in the liposomes acquiring substantial (30-fold over background) ribosome binding activity (Fig. 5a, column 4).

Quantification and stoichiometry

The process of ribosome binding to liposomes containing the 180K protein was saturable and Scatchard analysis indicated a dissociation constant (K_d) of $1.5 \times 10^{-8} \text{ M}$. This affinity is practically indistinguishable from the values of $1.9 \times 10^{-8} \text{ M}$ and $(1.2\text{--}2.0) \times 10^{-8} \text{ M}$ obtained for ribosome binding to canine pancreatic¹⁴ and rat liver^{13,17} microsomal membranes respectively. On the basis of the conclusion that the 180K species mediates the binding, these data can be used to determine the relative concentration of this receptor in the membrane, as well as the stoichiometry of the ribosome-receptor interaction. A functional quantification of ribosome binding to intact microsomes and to liposomes was made by assaying the number of ribosomes needed to saturate binding sites on both types of vesicles. When the amount of 180K receptor was quantified by densitometry, the ratio of ribosome-receptor, at saturation, was found to be the same. The result that there are 123 nmol ribosomes per g microsomal membrane protein, is in good agreement with the previously determined value of 161 nmol per g (ref. 14). A densitometric analysis of stained gels, using the M_r of 180K for the receptor, indicated that there are 127 nmol receptor per g membrane protein. We therefore conclude that the ribosome receptor comprises some 2–2.5% of total membrane protein, and that the ratio of ribosome-binding capacity to the number of receptors is virtually 1:1.

Discussion

The data presented here indicate that ribosome binding to the membranes of the rough ER is mediated by a large integral protein, the bulk of which is oriented towards the cytosol. This conclusion is supported by several lines of evidence from previous studies. An integral membrane protein with properties identical to those of the ribosome receptor is specifically localized in rough membranes from both rat liver¹⁸ and canine pancreas¹⁴. Other rough ER-specific proteins, including functionally characterized participants in translocation such as docking protein (SRP receptor) and signal peptidase, form a complex that is insoluble in nonionic detergents except at high salt concentrations¹⁹. A member of this complex is the ribosome receptor characterized here.

To our knowledge these experiments represent the first functional reconstitution of a purified membrane component of the translocation machinery into an artificial lipid bilayer. Previously, ribosome binding activity has been reconstituted into liposomes using a crude fraction of rat liver microsomal mem-

brane proteins characterized only by their inability to be bound by the plant lectin concanavalin A (ref. 20). This fraction contains a 180K protein (Fig. 3 in ref. 20). We have performed a similar experiment on the purified receptor and also come to the conclusion that it is not a glycoprotein (our unpublished observations). Several groups have succeeded in reconstituting protein translocation into lipid vesicles^{21,22}. Although the material incorporated into the liposomes in these experiments was still very heterogeneous, the protein profiles of these fractions consistently contained the 180K polypeptide that we conclude to be the ribosome receptor.

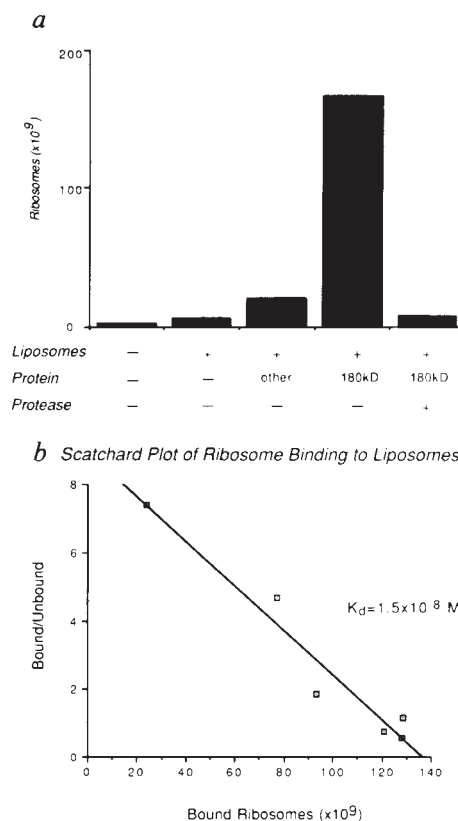


FIG. 5 a, Liposomes containing the 180K intact receptor bind ribosomes. Different membrane protein fractions were assembled into liposomes composed of phosphatidylserine and phosphatidylcholine. Ribosome binding assays were then carried out on the various types of liposomes, including: no liposomes (column 1); liposomes with no incorporated protein (column 2); liposomes with proteins depicted in Fig. 4, lane 7 (column 3); liposomes containing pure 180K receptor, as shown in Fig. 4, lane 8, (column 4); and the same liposomes as in column 4 that had been treated with protease (column 5). b, Scatchard analysis of the binding of ribosomes to the receptor-containing liposomes. Line was drawn by a least-squares linear-fit program. METHODS. The liposomes were prepared by a modification of the method of Gould-Fogerite and Mannino¹⁶. Fifty micrograms of the purified 180K receptor were added to 3 mg phosphatidylserine and 0.33 mg phosphatidylcholine (Sigma) in 200 mM CH_3COOK , 1.25% OG, 20% glycerol. This mixture was serially dialysed against stepwise-lowered concentrations of OG (in 50 mM KCl, 50 mM Tris-HCl, pH 7.5, 3 mM CaCl_2): 0.75% OG for 1 h at 4 °C; 0.3% OG for 4 h; and finally to buffer without OG for 16 h. The suspension was centrifuged at $100,000g$ for 20 min. The pellet was resuspended in 2.1 M sucrose in LSB, 2.5 mM EGTA, was placed in the bottom of a centrifuge tube, and overlaid with a step gradient of 1.9 M, 1.5 M and 0.25 M sucrose in LSB. The sample was centrifuged at $200,000g$ for 3 h. The liposomes were recovered from the 0.25 M/1.5 M sucrose interface and were used in a ribosome binding assay identical to that used for RM_{sn} (see Fig. 1), at a concentration of 80 ng protein per μl liposomes. Where applicable, liposomes were proteolysed with $2 \mu\text{g ml}^{-1}$ trypsin (Sigma) for 15 min at 0 °C, and the proteolysis stopped with an excess of soybean trypsin inhibitor (Sigma). Proteolysed liposomes were recovered by centrifugation in the Beckman airfuge for 15 min at 26 psi, and resuspended in 0.25 M sucrose in LSB.

Since the discovery that the granular surface of rough ER is a function of bound ribosomes, many different ER proteins have been proposed to be ribosome receptors, including cytochrome P_{450} (refs 23, 24), several membrane proteins defined only by their M_r s on acrylamide gels^{25,26} and ribophorins I and II (refs 27, 28). Although they copurify with known components of the translocation machinery, ribophorins I and II do not show the protease sensitivity exhibited by the ribosome-binding activity of ER membranes¹⁴. We incorporated a fraction enriched in ribophorins I and II (Fig. 4, lane 7) into liposomes and assessed its ability to bind ribosomes. It had only a marginal capacity for ribosome binding, probably due to the presence of a small amount of 180K receptor (Fig. 5a, column 3). Although these data do not rule out a role for ribophorins in translocation, it is now unlikely that they directly mediate the binding of ribosomes to the ER membrane.

During the synthesis of secretory proteins, ribosomes cycle

on and off the membrane²⁹. As it is unlikely that salt concentrations change dramatically at the surface of the ER membrane, there must be a way in which the attachment and detachment of ribosomes is regulated. The targeting to the rough ER and the initial interactions between the ER membrane and the nascent polypeptide chain are mediated by SRP and docking protein (SRP receptor)^{6,7,30}. Subunits of both SRP and docking protein have the consensus amino-acid sequence typical of GTP-binding proteins³¹⁻³³. GTP is required for the association of the translocation complex with the membrane³⁴. Although postulated to be involved with SRP receptor activity, the requirement for GTP could extend also to ribosome attachment and/or detachment *in vivo* (we have not observed a requirement for GTP in our *in vitro* attachment assay). Much should be learned about this once the primary structure of the receptor has been deduced and from genetic studies on its homologue in a genetically manipulable organism such as yeast. □

Received 1 June; accepted 6 July 1990.

- Palade, G. E. *Science* **189**, 347-358 (1975).
- Blöbel, G. & Dobberstein, B. *J. Cell Biol.* **67**, 835-851 (1975).
- Walter, P., Ibrahim, I. & Blöbel, G. *J. Cell Biol.* **91**, 545-550 (1981).
- Kurzchalia, T. V. et al. *Nature* **320**, 634-636 (1986).
- Walter, P. & Blöbel, G. *J. Cell Biol.* **97**, 1693-1700 (1983).
- Meyer, D. I., Krause, E. & Dobberstein, B. *Nature* **297**, 647-650 (1982).
- Gilmore, R., Walter, P. & Blöbel, G. *J. Cell Biol.* **95**, 470-477 (1982).
- Blöbel, G. & Sabatini, D. D. in *Biomembranes Vol. 2* (ed. Manson, L. A.) 193-195 (Plenum, New York, 1971).
- Hortsch, M. & Meyer, D. I. *Biol. Cell* **52**, 1-8 (1984).
- Walter, P., Gilmore, R. & Blöbel, G. *Cell* **38**, 5-8 (1984).
- Sabatini, D. D. & Blöbel, G. *J. Cell Biol.* **45**, 146-157 (1970).
- Mechler, B. & Vassalli, P. *J. Cell Biol.* **67**, 25-37 (1975).
- Borgese, N., Mok, W., Kreibich, G. & Sabatini, D. D. *J. molec. Biol.* **88**, 559-580 (1974).
- Hortsch, M., Avossa, D. & Meyer, D. I. *J. Cell Biol.* **103**, 241-253 (1986).
- Meyer, D. I., Louvard, D. & Dobberstein, B. *J. Cell Biol.* **92**, 579-583 (1982).
- Gould-Fogerite, S. & Mannino, R. J. *Analyt. Biochem.* **146**, 15-25 (1985).
- Shires, T. K. & Pitot, H. C. *J. Cell Biol.* **55**, 238a (1972).
- Amar-Costecec, A., Todd, J. A. & Kreibich, G. *J. Cell Biol.* **99**, 2247-2253 (1984).
- Hortsch, M., Crimando, C. & Meyer, D. I. in *Integration and Control of Metabolic Process: Pure and Applied Aspects* (ed. Kon, O. L. et al.) 3-11 (ICSU, Cambridge, 1987).
- Yoshida, H. et al. *Biochem. J.* **245**, 811-819 (1987).
- Yu, Y., Zhang, Y., Sabatini, D. D. & Kreibich, G. *Proc. natn. Acad. Sci. U.S.A.* **86**, 9931-9935 (1989).
- Nicchitta, C. V. & Blöbel, G. *Cell* **60**, 259-269 (1990).
- Ohlsson, R. & Jergil, B. *Eur. J. Biochem.* **72**, 595-603 (1977).
- Takagi, M. *J. Biochem. Tokyo* **82**, 1077-1084 (1977).
- Jothy, S., Bilodeau, J. L. & Simpkin, H. *Can. J. Biochem.* **53**, 1039-1045 (1975).
- Aulinskas, T. S. & Burden, T. S. *Hoppe-Seyler's Z. physiol. Chem.* **360**, 709-720 (1979).
- Kreibich, G., Ulrich, B. L. & Sabatini, D. D. *J. Cell Biol.* **77**, 464-487 (1978).
- Kreibich, G., Freinstein, C. M., Pereyra, B. N., Ulrich, B. L. & Sabatini, D. D. *J. Cell Biol.* **77**, 488-506 (1978).
- Borgese, D., Blöbel, G. & Sabatini, D. D. *J. molec. Biol.* **74**, 415-438 (1973).
- Gilmore, R. & Blöbel, G. *Cell* **35**, 677-685 (1983).
- Connolly, T. & Gilmore, R. *Cell* **57**, 599-610 (1989).
- Römsch, K. et al. *Nature* **340**, 478-482 (1989).
- Bernstein, H. D. et al. *Nature* **340**, 482-486 (1989).
- Connolly, T. & Gilmore, R. *J. Cell Biol.* **103**, 2253-2261 (1986).
- Blöbel, G. & Dobberstein, B. *J. Cell Biol.* **67**, 852-862 (1975).
- Matson, R. S. & Siebert, C. *Preparative Chromatography* **1**, 67-72 (1988).
- Anson, W. J. *biochem. biophys. Meth.* **11**, 13-20 (1985).
- Bradford, M. M. *Analyt. Biochem.* **72**, 248-254 (1976).
- Fujiki, Y., Hubbard, A. L., Fowler, S. & Lazarow, P. B. *J. Cell Biol.* **93**, 97-102 (1982).
- Hortsch, M., Avossa, D. & Meyer, D. I. *J. biol. Chem.* **269**, 9137-9145 (1985).
- Sobue, K. et al. *Proc. natn. Acad. Sci. U.S.A.* **85**, 482-489 (1988).
- Fukui, Y., Yumura, S. & Yumura, T. K. *Meth. Cell Biol.* **28**, 347-356 (1987).

ACKNOWLEDGEMENTS. We thank N. Kedersha for carrying out the immunofluorescence studies, D. Nicolas for initial efforts in liposome construction, M. Hortsch for his PhD thesis and card file, and to G. S. Payne for critical advice. A.J.S. is supported by the Medical Scientist Training Program (USPHS), D.J.M. by the American Cancer Society. This research was funded by the USPHS, the Cancer Research Coordinating Committee of the University of California, and the Weingart Foundation.

LETTERS TO NATURE

X-ray evidence of an obscured nucleus in the type 2 Seyfert galaxy Mkn3

H. Awaki, K. Koyama, H. Kunieda & Y. Tawara

Department of Astrophysics, School of Science, Nagoya University, Chikusa-ku, Nagoya 464-01, Japan

SEYFERT galaxies comprise the majority of active galactic nuclei (AGNs), in which a bright compact nucleus is thought to be powered by matter accreting onto a massive black hole. Seyfert galaxies are classified as type 1 or 2 according to the presence or absence of broad emission lines in the optical spectrum. The high velocities indicated by the broad lines in Seyfert 1 galaxies are taken to be good evidence of a compact, massive object, as are the strong and variable hard X-ray sources that are also generally observed in these objects. In contrast, Seyfert 2 galaxies possess neither of these characteristics, so the theory that they too have an accreting massive black hole is less compelling. Since the discovery¹ by spectropolarimetry of a 'hidden' Seyfert 1 nucleus in the prototypical Seyfert 2, NGC1068, the long-standing hope that the two classes may be unified has been revived. Here we present evidence

from observations by the Ginga satellite that another Seyfert 2, Mkn3, has the X-ray spectral signature of a hidden type 1 nucleus.

Optical spectropolarimetry of NGC1068 revealed faint, polarized broad emission lines characteristic of a Seyfert 1 galaxy¹. A model in which the broad-line emitting region is obscured from view by a thick torus also explains the polarized flux as emission scattered into our line of sight by electrons in a tenuous, warm gas above and below the axis of the torus. If this model is generally applicable, any Seyfert 1 could look like a Seyfert 2 if the inclination angle of the torus prevents a clear view of the nucleus. Hard X-ray data on NGC1068² also support this model, as an unusually large observed equivalent width of the iron K α line naturally arises from fluorescence in the warm scattering region, whereas the direct continuum is blocked from our view by the torus, which has a hydrogen column density of $>10^{25} \text{ cm}^{-2}$. Further Ginga observations of Seyfert 2 galaxies have revealed hard X-rays from Mkn348³ and NGC4388⁴ with absorbing column densities of $\sim 10^{23} \text{ cm}^{-2}$. This is not very large when compared with some type 1 galaxies, so the general applicability of this model is still uncertain.

Another Seyfert 2, Mkn3, was observed by the Large Area Counter (LAC) onboard the Ginga satellite on 25-26 September 1989. The LAC consists of eight identical proportional counters, sensitive in the 1.5-38 keV band, with a total effective area of $4 \times 10^3 \text{ cm}^2$ (ref. 5). Scanning observations detected excess

TABLE 1 Results of spectral fit

Flux 2–10 keV (erg s ⁻¹ cm ⁻²)	Photon index α	Column density log (N_{H} (cm ⁻²))	Equivalent width (eV)	Energy* (keV)	log (N_{HFe} (cm ⁻²))†	Energy‡ (keV)	χ^2 §
1.0×10^{-11}	1.3 ± 0.3	$23.77^{+0.06}_{-0.08}$	540^{+120}_{-50}	6.45 ± 0.1	$23.80^{+0.16}_{-0.17}$	$7.45^{+0.2}_{-0.3}$	22.8

Errors are quoted at the 90% confidence level.

* Energy of iron line corrected for the redshift.

† $N_{\text{HFe}} = 10^{4.48} N_{\text{Fe}}$.

‡ Energy of iron edge corrected for the redshift.

§ 19 degrees of freedom.

emission at the position of Mkn3. A pointing observation with a 1.2×10^4 -s exposure was then made, from which the X-ray spectrum of Mkn3 was derived. Figure 1 shows the pulse-height spectrum after background subtraction. We fitted the spectrum using a standard power-law model and a term to account for absorption by cold gas. An emission line of iron, which is often detected in AGNs, was also included in the fitting procedure. This model was acceptable with the best-fit parameters listed in Table 1. The photon index, 1.3 ± 0.3 , and the intrinsic 2–10-keV luminosity of 4×10^{43} erg s⁻¹ (at a distance of 79 Mpc), are within the range of typical type 1 galaxies. The absorption corresponds to a hydrogen column density of $(6^{+3}_{-2}) \times 10^{23}$ cm⁻², the largest yet measured for a Seyfert galaxy. The edge energy, after correction for a redshift of 0.0132, is consistent with that from iron with an ionization state less than Fe(XVI). In this case, the K shells of carbon and oxygen are expected to be nearly filled⁶, and the low-energy absorption that arises in these lighter elements is consistent with the depth of the iron K edge for cosmic abundances of cold matter.

A Compton scattering model was also tried and rejected, because χ^2 was 387 for 22 degrees of freedom. This implies that the low-energy turnover, as well as the iron edge structure at ~ 7 keV, is due to simple absorption by cold gas with cosmic abundance.

Our interpretation of this spectrum is that Mkn3 is a case intermediate between less-observed type 2 Seyferts such as Mkn348 and completely absorbed nuclei like that of NGC1068. In terms of the torus model, there is a more favourable viewing angle (lower inclination) toward the nucleus of Mkn3, or possibly a torus with a smaller column density than that of NGC1068.

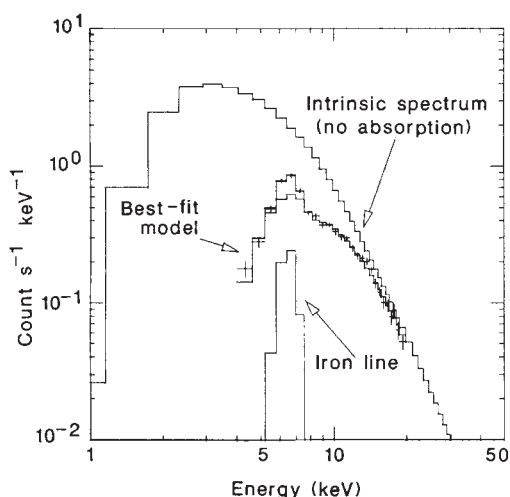


FIG. 1 The LAC pulse-height spectrum measured for Mkn3. The histograms show the best-fit model as described in the text and the intrinsic spectrum of Mkn3 without absorption. The intrinsic spectrum is similar to that of type 1 Seyferts in this energy range.

The soft X-ray detection of Mkn3 by the Einstein imaging proportional counter is consistent with the model if the soft X-rays belong to the scattered component. The 0.5–4.5-keV luminosity of 6.4×10^{41} erg s⁻¹ (ref. 7) is almost two orders of magnitude lower than expected from extrapolation of the intrinsic (unabsorbed) Ginga spectrum. This is as expected if the scattered component is $\sim 1\%$ of the direct flux. Because the column density derived from the X-ray data is extremely high, both the direct soft X-rays and broad optical emission lines are blocked from our view. Consistent with this picture, broad emission lines have been detected in the polarized flux from Mkn3 (ref. 8).

If the heavily absorbed X-ray spectrum of Mkn3 is more or less typical among Seyfert 2 galaxies, it would explain the weak detections or upper limits on the soft X-ray flux from the Einstein Observatory. Seyfert 2 galaxies generally have soft X-ray luminosities $< 10^{42}$ erg s⁻¹, whereas type 1 galaxies have luminosities in the range 10^{42} – 10^{44} erg s⁻¹ (ref. 9). Further hard X-ray observations of Seyfert 2 galaxies may reveal, as in the case of Mkn3, that the hard X-ray luminosities of the two classes are similar. Hard X-rays are the crucial test, because a gas of column density 10^{23} – 10^{24} cm⁻² is transparent above several keV, but highly absorbing in the soft X-ray and optical bands.

This result could also have important implications for the origin of the cosmic X-ray background. Because the number of Seyfert 2 galaxies is estimated¹⁰ to be several times larger than that of type 1, their contribution to the hard X-ray background might not be negligible. If the contribution of high-luminosity AGNs such as type 1 Seyferts and quasars is removed, there is a low-energy turnover in the spectrum of the residual cosmic X-ray background¹¹. This turnover is in agreement, at least qualitatively, with the low-energy absorption feature observed in Mkn3. The spatial distribution of the cosmic X-ray background determined from the Ginga data suggests that the mean X-ray spectrum of unresolved AGNs has a larger column density than that of typical high-luminosity AGNs^{12,13}.

A more quantitative discussion of these issues requires further systematic hard X-ray observations of Seyfert 2 galaxies, which will be a major project for Ginga, as well as for the Astro-D satellite (Japan), XMM (X-ray Multi Mirror) (European Space Agency) and XTE (X-ray Timing Explorer) (NASA). □

Received 21 March; accepted 18 June 1990.

- Antonucci, R. R. J. & Miller, J. S. *Astrophys. J.* **297**, 621–632 (1985).
- Koyama, K. et al. *Publ. astr. Soc. Jap.* **41**, 731–737 (1989).
- Warwick, R. S. et al. *Publ. astr. Soc. Jap.* **41**, 739–744 (1989).
- Takano, S. et al. *Astrophys. J.* (submitted).
- Turner, M. J. L. et al. *Publ. astr. Soc. Jap.* **41**, 345–372 (1989).
- Kallman, T. R. & McCray, R. *Astrophys. J. Suppl.* **501**, 263–318 (1982).
- Kriss, G. A. thesis, Mass. Inst. Technol. (1982).
- Miller, J. S. & Goodrich, R. W. *Astrophys. J.* **355**, 456–467 (1990).
- Kriss, G. A. et al. *Astrophys. J.* **242**, 492–501 (1980).
- Osterbrock, D. E. & Shaw, R. A. *Astrophys. J.* **327**, 89–98 (1988).
- Giacconi, R. & Zamorani, G. *Astrophys. J.* **313**, 20–27 (1987).
- Hayashida, K. thesis, Tokyo University (1990).
- Warwick, R. S. & Stewart, G. C. *Proc. 23rd ESLAB Symp. 'X-ray Astronomy'* Vol. 2 (eds Hunt, J. & Battrick, B.) 727–732 (European Space Agency, Paris, 1989).

ACKNOWLEDGEMENTS. We thank all members of the Ginga team and J. Halpern, K. Leighly and M. A. Lim for reading the manuscript. The data analysis was performed on Facom M380 computer of the high-energy physics laboratory of Nagoya University.

Triton's streaks as windblown dust

Carl Sagan & Christopher Chyba

Laboratory for Planetary Studies, Cornell University, Ithaca, New York 14853, USA

THE encounter of the Voyager 2 spacecraft with Neptune's satellite Triton revealed many 'dark' (about 10–20% darker than the adjacent frost) surface streaks in Triton's southern hemisphere¹, resembling the streaks that are due to windblown dust on Mars². It seems therefore that dust transport by winds in Triton's tenuous atmosphere is required, the main question being the mechanism for raising dust from the surface or sub-surface. The two obvious candidates are geyser-like eruptions and direct lofting by surface winds¹. Here we show that, despite Triton's tenuous ($16 \pm 3 \mu\text{bar}$) atmosphere³, low-cohesion grains with diameters of $\leq 5 \mu\text{m}$, may be carried into suspension by aeolian surface shear stress, given expected geostrophic wind speeds⁴ of $\sim 10 \text{ m s}^{-1}$. (The wind velocities needed to lift grains as cohesive as those found on Earth, however, are implausibly high.) For erupting plumes, we show that dust-settling timescales and expected wind velocities yield streak length scales in good agreement with those observed. Both candidate mechanisms therefore seem to be consistent with present observations of Triton.

Ground-based observations of seasonal and secular albedo changes on Mars were explained by Sagan and Pollack^{5,6} in terms of windblown dust. Based on the classic study by Bagnold⁷, they showed that the threshold critical velocity to initiate grain motion at the base of the martian boundary layer is consistent with plausible wind velocities on Mars. (We call all such transport—whether the particles maintain their ballistic trajectories or are entrained by winds—saltation, from the Latin word for 'jump'.) Subsequent Mariner 9 and Viking data revealed time-variable dark irregular 'splotches', and bright and dark 'streaks' typically tens of kilometres long, many emanating from impact craters^{2,8,9}. Martian general circulation and topographic slope winds generally seem strong enough, and in the proper directions, to account for the wind streaks^{2,9}.

The dependence of threshold friction velocity (the minimum wind speed required for saltation) on particle diameter D_p is usually taken to be roughly parabolic⁷. The velocity increases to large D_p because drag scales as the area but the mass scales as the volume. At lower diameters, the increase of threshold velocity with decreasing D_p was also thought, from limited data, to be a fluid mechanics effect⁷. But subsequent work^{10–14}—including data on cohesion-free particle transport in fluid beds—indicates that the effect is due to cohesion, which increases the effective weight of small grains.

Although the surface pressure on Triton is only $\sim 0.25\%$ that on Mars, dark streaks of a remarkably martian aspect are observed on Triton's southern summer polar cap. Like many martian streaks, those on Triton are mainly tens of kilometres long. Although association with impact craters, rare in the Triton polar cap, is not generally evident, Triton streaks frequently emanate from dark splotches a few kilometres across (and, more rarely, from small bright regions)¹.

The mass loading of Triton's atmosphere is $P/g \approx 0.2 \text{ g cm}^{-2}$ (where $P = 16 \pm 3 \mu\text{bar}$ nitrogen and $g = 78 \text{ cm s}^{-2}$ are Triton's surface pressure³ and gravitational acceleration, respectively), and the amount of ice (presumably nitrogen and methane) deposited on the polar cap in winter is probably more than enough to cover over any preexisting streaks. Such streaks might be exposed again in the following spring and summer, but it is also possible that new streaks are produced in the caps once each seasonal cycle (~ 165 Earth years long).

A plausible source of the (relatively) dark material on Triton is complex organic heteropolymers produced by neptunian

magnetospheric electrons, cosmic rays and solar ultraviolet radiation¹⁵. These organics might be formed in the atmosphere, or directly on the surface from methane and other hydrocarbon ices^{15,16}. An origin from processes inside Triton, such as ice volcanoes or fumaroles, is also possible¹. This would readily explain the association of streaks with particular small source regions; particles could then be transported either in suspension by the prevailing winds at altitude, or by saltation and subsequent entrainment by winds nearer the surface. A rosette diagram of the directions of wind streaks on Triton¹⁷ shows preferred orientations approximately consistent with those given by Ingersoll's⁴ general circulation model. If the dark material is generated by radiation in the atmosphere, its arrival at the surface in small particles would be easily understood, but its concentration in source regions would require preferential trapping in topographic features below the resolution limit, as is thought to occur on Mars (in crater floors, for example^{2,8}).

The threshold wind velocity u_{*0} to initiate motion of particles of diameter D_p and density ρ_p where the atmospheric density is ρ is given by⁷

$$u_{*0} = A(D_p g \rho_p / \rho)^{1/2} \quad (1)$$

where the Bagnold parameter A is a dimensionless constant that is a measure of the ratio of the mean fluid shear stress to the gravity stress on the topmost layer of dust grains; typically, $A \approx 0.1$. Equation (1) can be derived within the factor A by equating the gravitational force mg on a particle of mass m to the dynamic wind pressure ρu_{*0}^2 acting on the cross-sectional area of the particle. Traditionally⁷, A has been taken to be a function only of B , the frictional Reynolds number, $B = D_p u_{*0} / \nu$, where ν is the kinematic viscosity. But fluid-bed and wind-tunnel experiments show that for small particles with $B \leq 1$, A is a function not only of B , but also of interparticle forces^{10–14}.

Surface gravity and atmospheric pressure on Triton are in good agreement with an assumption of vapour-pressure equilibrium at a surface temperature¹⁸ of 38^{+3}_{-4} K . From the ideal-gas law, $\rho = 1.4 \times 10^{-7} \text{ g cm}^{-3}$. Mars has $g = 371 \text{ cm s}^{-2}$ and $P \approx 6 \text{ mbar CO}_2$, giving $\rho = 1.5 \times 10^{-5} \text{ g cm}^{-3}$ for a surface temperature¹⁹ of 215 K . Dust densities on Mars are probably those appropriate to sand^{7,11}, $\sim 2.7 \text{ g cm}^{-3}$. Dust on Triton is probably a combination of methane or nitrogen ice and dark organics¹. Densities for methane and nitrogen ices are 0.5 g cm^{-3} and 0.9 or 1.0 g cm^{-3} , respectively²⁰, with the latter density determined by whether nitrogen is present in its α or β form [the α - β phase transition occurs at 35.6 K (ref. 21 and R. H. Brown, personal communication)]. Typical densities for condensed simple organics are ~ 0.5 – 0.7 g cm^{-3} , and for more complex organics²² are 0.7 – 1.2 g cm^{-3} . We take $\rho_p = 0.75 \text{ g cm}^{-3}$ as a typical dust density on Triton.

The ratio of the threshold wind velocity for particle motion on Triton to that on Mars is then, from equation (1),

$$u_{*0}^T / u_{*0}^M = 2.5(A^T/A^M)(D_p^T/D_p^M)^{1/2} \quad (2)$$

where the superscripts T and M refer to Triton and Mars. Equation (2) shows that for particles of a given diameter, the threshold wind velocity on Triton is only ~ 2.5 times higher than that on Mars, assuming that the value of the Bagnold parameter is the same.

Given the extremely low atmospheric pressures of Triton ($P^M/P^T \approx 400$), these results seem counterintuitive; but there are four factors that mitigate the effect of low pressure: Triton's surface gravity is lower than that of Mars by a factor of ~ 5 , dust densities on Triton are probably lower by a factor of ~ 3 , u_{*0} depends on the density, not the pressure, ratio (atmospheric density depends inversely as temperature, and Triton's temperature is a factor of ~ 6 lower than that of Mars), and u_{*0} has only a square-root dependence on all of these parameters.

What is u_{*0} for Triton? First we must determine B . Ingersoll⁴ has used scaling arguments to obtain a kinematic viscosity $\nu = 450 \text{ cm}^2 \text{ s}^{-1}$. Given this value, even particles with D_p as large as 0.1 cm will have $B \ll 1$ for any plausible u_{*0} (see below), so that we are in the highly laminar regime where A depends on both B and D_p . Iversen *et al.*¹¹ fit an analytical model for u_{*0} incorporating drag and lift, rotational moment of inertia, weight and interparticle forces to a variety of wind-tunnel data, including experiments in the regime of very low air density and Reynolds number. These latter experiments were greatly extended by Iversen and White¹³. Greeley and Iversen¹⁴ fit these data, for $B \leq 0.3$, with an expression like equation (1), but with the coefficient A given by

$$A \equiv A(B, D_p) = 0.2[(1 + I_p/\rho_p g D_p^3)/(1 + 2.5B)]^{1/2} \quad (3)$$

where the interparticle force I_p is found to be $0.006 D_p^{1/2}$. From equations (3) and (1), we determine u_{*0} as a function of D_p , for the case where Triton dust is subject to interparticle forces (cohesion) comparable to those between terrestrial grains.

We also consider the possibility that interparticle cohesion on Triton is much weaker than on Earth (or on Mars, or on the Moon). Cohesion owing to thin adsorbed layers of liquid water, important on Earth^{13,23,24}, is certainly missing on Triton. The cohesion of lunar grains is greater than that of terrestrial grains²⁵, partly owing to agglutination from micrometeorite impacts²⁶ and partly to vacuum sintering¹⁴. Both effects may be weakened in the thin atmosphere of Triton. There is some (weak) spectroscopic evidence for water ice at Triton's surface²⁷, in which case hydrogen bonding might act as an interparticle cohesive force. In the absence of significant surficial water ice, perhaps only van der Waals forces²⁸ remain effective. We therefore analyse the two extremes—one in which cohesion is comparable to that of Earth, and the other in which there is no cohesion at all.

A number of investigators have considered the cohesionless regime^{10–14}, and it has been invoked to avoid having to postulate Mach 0.5 winds on the slopes of Pavonis Mons on Mars²⁹. In this case, u_{*0} is given by equations (1) and (3) with I_p set equal to zero. In Fig. 1, we show aeolian threshold frictional velocities for particle motion on Triton for both cohesive and cohesionless grains, as a function of D_p . For cohesive grains, threshold velocities of at least $\sim 10 \text{ m s}^{-1}$ are required for particle motion. In the cohesionless case, however, velocities as low as ~ 0.4 – 1.0 m s^{-1} will be sufficient to loft ~ 1 – $10 \text{ }\mu\text{m}$ grains.

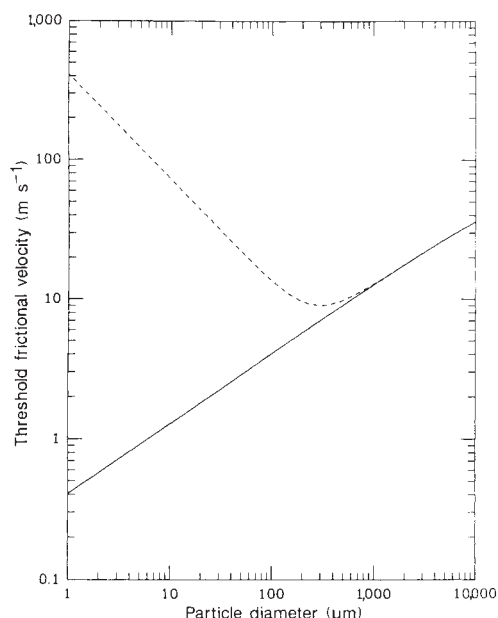


FIG. 1 Threshold velocity as a function of particle radius for cohesive (dashed line) and cohesionless (solid line) dust particles on Triton.

Can friction velocities u_* of this magnitude occur at Triton's surface? Ingersoll⁴ has studied the dynamics of Triton's atmosphere, and finds wind speeds v at the top of a ~ 1 -km boundary layer to be of the order of Triton's tangential speed of rotation, 16.7 m s^{-1} . The friction velocity should be related to v according to $v^2/u_*^2 = 1/C_D$, where the drag coefficient $C_D \approx 0.002$ (ref. 4). This approach gives $u_* \approx 0.75 \text{ m s}^{-1}$.

A second approach, given v at the top of the boundary layer, is to adopt the standard Prandtl logarithmic variation of wind velocity with distance z above the surface^{14,24}

$$v = (u_*/k) \ln(z/z_0) \quad (4)$$

where $k = 0.4$ is the von Kármán constant and z_0 is the roughness length, a function of both Reynolds number and the shape of and distance between surface-roughness elements; typical prescriptions for z_0 range from $D_p/30$ for rough surfaces¹⁴ to $\nu/30u_*$ for small B (ref. 12). Because of the logarithmic dependence in equation (4), u_* is insensitive to z_0 . Taking z to be the height of the boundary layer⁴ ($\sim 1 \text{ km}$), equation (4) with the above choices of z_0 gives $u_* \approx 0.3$ – 0.4 m s^{-1} , in rough agreement with the calculation based on C_D . Thus, surface friction velocities $u_* \approx 0.1$ – 1.0 m s^{-1} seem probable. Figure 1 therefore shows that saltation is possible for cohesionless grains with diameters $D_p \leq 5 \text{ }\mu\text{m}$. If $z_0 \gg 5 \text{ }\mu\text{m}$, however, such grains might typically lie entirely within the laminar sub-layer below z_0 , within which wind velocities could be much less than u_* (ref. 14). The required threshold wind velocities for cohesive grains are an order of magnitude too high. Unless wind gusts within $\sim 100 \text{ }\mu\text{m}$ of the surface occasionally reach $\sim 10 \text{ m s}^{-1}$, about the value at the top of the boundary layer, grains as cohesive as silicate particles on Earth cannot be set into motion by Triton's winds. A possible exception would be entrainment initiated by bombardment of grains on Triton's surface by dust falling from erupted plumes. On Earth, such impact-induced entrainment can maintain movement of cohesive grains for wind velocities at or below those sufficient for initiating movement of cohesionless grains^{7,14,24}.

Finally, we compare u_* with the terminal velocity V (derived in equation (5) below) of a settling particle. Evidently $V < u_*$ for any value of u_* capable of lifting a particle of diameter D_p . This implies that any grain lofted from Triton's surface would be placed into suspension, rather than moving in low, multiple hops, which occurs only when $V \geq u_*$ (ref. 14).

Another potential mechanism for producing streaks is geyser-like eruptions¹ which inject dust into the prevailing winds (possibly analogous to the formation of elongate albedo markings on Io³⁰). The fact that the surface streaks observed between 10° and 30° S trend towards the northeast implies that they are controlled by winds in the boundary layer, that is, within $\sim 1 \text{ km}$ of the surface⁴. This, then, will be our choice of injection height h for calculating the effects of wind velocities and fallout timescales for injected dust. Poleward of 30° S , however, streak directions become more irregular¹. Similar dark streaks with diffuse downwind boundaries will be produced by settling dust whether it is geysered or saltated up into suspension.

Dust settling may occur in the Stokes (viscous) regime, when the gas mean free path $\lambda \ll D_p$, or in the Epstein (kinetic) regime, when $\lambda \gg D_p$. The mean free path is $(\sqrt{2}n\sigma)^{-1}$, where n is the number density and σ the collision cross-section of the gas. For Triton's nitrogen atmosphere, $n = 3.1 \times 10^{15} \text{ cm}^{-3}$, and $\sigma = 4.3 \times 10^{-15} \text{ cm}^2$ (ref. 31), giving $\lambda = 530 \text{ }\mu\text{m}$ ($16 \text{ }\mu\text{bar}/P$). Thus, settling dust on Triton is in the Epstein regime when $D_p \leq 100 \text{ }\mu\text{m}$. Settling speed V in the Epstein regime may be calculated by equating the gravitational force on a dust particle, mg , to the buoyancy force F exerted by the atmosphere, assuming no convective transport. For a spherical dust particle, $F = (\pi/3)\delta\bar{v}\rho D_p^2 V$, where $\bar{v}$ is the mean molecular speed, and δ is a constant that depends on the law of reflection of gas molecules from the surface of a sphere^{32,33}. For specular reflection, $\delta = 1.0$, and for diffuse reflection $\delta = 1.4$. As the latter value is in good agreement with experiment^{32,33}, we adopt it here. Equating F

and mg yields $V = 0.36 D_p \rho_p g / \rho \bar{v}$, which for 10- μm particles becomes

$$V = 8.9 (D_p / 10 \mu\text{m}) (16 \mu\text{bar} / P) \text{ cm s}^{-1} \quad (5)$$

Thus a 10- μm particle will settle out from an injection height $h = 1 \text{ km}$ in $\sim 3 \text{ h}$.

The length of the dark streaks on Triton range from several tens to about one hundred kilometres¹. Taking a characteristic length $L = 50 \text{ km}$, we find the required wind velocity in the boundary layer to be

$$v = L(h/V) = (L/50 \text{ km})(1 \text{ km}/h)(D_p/10 \mu\text{m}) 4.5 \text{ m s}^{-1}$$

Ingersoll⁴ estimates typical wind velocities in the boundary layer to be $\sim 10 \text{ m s}^{-1}$. Settling timescales and expected wind velocities in the boundary layer therefore yield streak length scales in good agreement with those observed. Larger particles will fall out closer to the source regions and smaller particles farther. Impacts by dust settling from such eruptions could then also initiate motion of surface grains.

If geysers or fumaroles loft significant amounts of dark dust in the boundary layer, it seems that the streaks can be well understood without any further hypotheses. This would, however, require a large number of geysers to have erupted, perhaps on as short a timescale as the $\sim 165\text{-yr}$ seasonal cycle. The discovery¹ of two or three such eruptions during Voyager 2's few-hour flyby of Triton suggests that this is not a very stringent condition. Indeed, for hundreds of streaks to form on Triton over $\sim 165 \text{ yr}$, on average several must erupt in any given year. The Voyager results then suggest that a typical geyser erupts for $\sim 1 \text{ yr}$ out of every 100, although these eruptions need not be continuous. (Moreover, we do not know whether Voyager's observation of two or three erupting plumes was typical of Triton, nor did Voyager image all of Triton's surface.) Consider a large dark streak, formed by such an eruption, $\sim 100 \text{ km}$ long and 10 km wide. A bright ice surface will be significantly darkened by an admixture of as little as 0.1–1.0% of dark dust³⁴. Therefore $\sim 1\text{--}10 \text{ km}^2$ of dark dust is required to form the streak. For an average particle radius $\sim 1 \mu\text{m}$, this corresponds to $\sim 10^5\text{--}10^6 \text{ g}$ of dust. Thus to form a large dark streak, an eruption rate of dark dust as low as $\sim 0.01\text{--}0.1 \text{ g s}^{-1}$ for the 1-yr eruption period is required. A question for future research is whether geysers and saltation generate streaks with distinguishable characteristics. $\square$

32. Epstein, P. S. *Phys. Rev.* **23**, 710–733 (1924).

33. Kennard, E. H. *Kinetic Theory of Gases* (McGraw-Hill, New York, 1938).

34. Clark, R. *Icarus* **49**, 244–257 (1982).

ACKNOWLEDGEMENTS. We thank R. H. Brown, P. Gierasch, C. Hansen, A. Ingersoll, T. V. Johnson, J. B. Pollack, J. Schwartz, L. Soderblom, P. C. Thomas and W. R. Thompson for discussions and suggestions. This work was supported by NASA.

Anomalous H^+ and D^+ conductance in H_2O – D_2O mixtures

H. Weingärtner* & C. A. Chatzidimitriou-Dreismann†‡

* Institut für Physikalische Chemie und Elektrochemie, Universität Karlsruhe, Kaiserstrasse 12, D-7500 Karlsruhe, FRG

† I. N. Stranski-Institut für Physikalische und Theoretische Chemie, Technische Universität Berlin, Strasse des 17. Juni 112, D-1000 Berlin 12, FRG

A KNOWLEDGE of proton-transfer dynamics and hydrogen-bonding in water and aqueous solutions is necessary for the understanding of many important chemical and biological processes. For example, quantum effects related to proton transfer (or tunneling) in $\text{H}^+(\text{H}_2\text{O})_n$ clusters of liquid water (where $n = 1, 2, \dots$) are known to have a dominant role in the proton conductance mechanism^{1,2} and are responsible for the high conductances of H^+ and OH^- in water. A new quantum theoretical approach to this process has been presented³, which is based on the hypothesis that there are quantum correlations^{4–8} between each H^+ and the protons of the surrounding water molecules, leading to the formation of coherent dissipative structures^{3,8}. From further investigations, one of us predicted that an anomalous decrease of H^+ conductance in H_2O – D_2O mixtures would take place⁹. Having thought of an experiment to test these predictions⁹ we now report the experimental results and conclude that an anomalous decrease in proton conductance does indeed occur.

The aforementioned experiment consists in the measurement of molar conductances of different HCl–DCl and KCl solutions in H_2O – D_2O mixtures. The experimental set-up, the results and their analysis are as follows.

For solutions in normal water, we used deionized and distilled H_2O with a specific conductance of $\sim 1 \times 10^{-6} \text{ S cm}^{-1}$. A stock solution of HCl in H_2O with a molar concentration of $\geq 0.5 \text{ mol dm}^{-3}$ was prepared, the exact concentration of which was determined by potentiometric titration of H^+ against NaOH and of Cl^- against AgNO_3 . The concentration of this solution was then adjusted to 0.5 mol dm^{-3} (error $< \pm 0.05\%$). The conductance of the adjusted stock solution agreed with the value interpolated from the data of Stokes¹⁰. Using density values from ref. 11, other solutions were obtained by weight dilution of the stock.

Solutions of DCl in D_2O were prepared using the same procedure. The D_2O (deuterium content $> 99.99 \text{ atom } \%$, IC-Chemikalien, München) had a specific conductance of $< 2 \times 10^{-6} \text{ S cm}^{-1}$, and was used without further purification. DCl was obtained as a 36 wt% solution in D_2O (D content $> 99.9\%$). We used the density of pure D_2O given in ref. 12, and assumed the partial molar volume of HCl to be the same in H_2O and D_2O , which allowed us to convert from weight per cent to molarity. This assumption was verified by some representative density measurements with an Anton Paar vibrating-tube densimeter (accuracy, $\pm 2 \times 10^{-5} \text{ g cm}^{-3}$).

Mixed solutions of the desired deuterium content (D atom fractions $X_D = 0.25, 0.5$ and 0.75) were prepared by mixing HCl– H_2O solutions with DCl– D_2O solutions of the same molarity. The same procedure was applied for obtaining solutions of KCl in H_2O – D_2O mixtures. The small excess volume of mixing

Received 14 March; accepted 18 June 1990.

- Smith, B. A. *et al. Science* **246**, 1422–1449 (1989).
- Sagan, C. *et al. Icarus* **17**, 346–372 (1972).
- Tyler, G. L. *et al. Science* **246**, 1466–1472 (1989).
- Ingersoll, A. P. *Nature* **344**, 315–317 (1990).
- Sagan, C. & Pollack, J. B. *Smithsonian Astrophys. Obs. Spec. Rep.* 255 (1967).
- Sagan, C. & Pollack, J. B. *Nature* **223**, 791–794 (1969).
- Bagnold, R. A. *The Physics of Blown Sand and Desert Dunes* (Chapman & Hall, London, 1954).
- Sagan, C. *et al. J. Geophys. Res.* **78**, 4163–4196 (1973).
- Thomas, P., Veeverka, J., Lee, S. & Bloom, A. *Icarus* **45**, 124–153 (1981).
- Sagan, C. & Bagnold, R. A. *Icarus* **26**, 209–218 (1975).
- Iversen, J. D., Pollack, J. B., Greeley, R. & White, B. R. *Icarus* **29**, 381–393 (1976).
- Pollack, J. B., Haberle, R., Greeley, R. & Iversen, J. *Icarus* **29**, 395–417 (1976).
- Iversen, J. D. & White, B. R. *Sedimentology* **29**, 111–119 (1982).
- Greeley, R. & Iversen, J. D. *Wind as a Geological Process* (Cambridge University Press, 1985).
- Thompson, W. R., Murray, B., Khare, B. N. & Sagan, C. *J. Geophys. Res.* **92**, 14933–14947 (1987).
- Khare, B. N. *et al. Icarus* **79**, 350–361 (1989).
- Hansen, C. *et al. Geophys. Res. Lett.* (submitted).
- Conrath, B. *et al. Science* **246**, 1454–1458 (1989).
- Carr, M. H. *The Surface of Mars* (Yale University Press, 1981).
- Simonelli, D. P. *et al. Icarus* **82**, 1–35 (1989).
- Prokhorov, A. I. & Yantsevich, L. D. *Soviet J. low-temp. Phys.* **9**, 94–97 (1983).
- Sagan, C., Thompson, W. R. & Khare, B. N. in *The Search for Extraterrestrial Life* (ed. Papagiannis, M. D.) 107–121 (Reidel, Boston, 1985).
- Gregg, S. J. *The Surface Chemistry of Solids* Ch. 3 (Chapman & Hall, London, 1961).
- Pye, K. *Aeolian Dust and Dust Deposits* Ch. 3 (Academic, London, 1987).
- Mitchell, J. *et al. Proc. Third Lunar Sci. Conf.* Vol. 3 (ed. Criswell, D. R.) 3235–3253 (MIT Press, Cambridge, 1972).
- Lindsay, J. F. *Lunar Stratigraphy and Sedimentology* Ch. 6 (Elsevier Scientific, New York, 1976).
- Cruikshank, D. P., Brown, R. H. & Clark, R. N. *Icarus* **58**, 293–305 (1984).
- Kitchener, J. A. in *Powders in Industry* 405–410 (Soc. chem. Ind., London, 1961).
- Sagan, C. *et al. Icarus* **22**, 24–47 (1974).
- Lee, S. W. & Thomas, P. C. *Icarus* **44**, 280–290 (1980).
- Atkins, P. W. *Physical Chemistry* (Freeman, New York, 1986).

‡ Correspondence can be addressed to either author.

was assumed to be the same as for salt-free H₂O–D₂O mixtures¹³.

Electrical conductances were measured with a Wayne–Kerr B-905 bridge as described elsewhere¹⁰. We used cells of a design described in ref. 14; the cell constants ranged from 30 to 300 cm⁻¹. Duplicate measurements in cells with different cell constants and at different frequencies (400–10,000 Hz) agreed to better than $\pm 0.05\%$. The overall precision of the conductance measurements is $\pm 0.05\%$. All data are based on Jones–Bradshaw standards¹⁴ converted from (international Ω)⁻¹ cm⁻¹ to (absolute Ω)⁻¹ cm⁻¹, that is, to S cm⁻¹.

Table 1 presents the experimental results for the molar conductance Λ of HCl–DCl in H₂O–D₂O mixtures at 25 °C. We have retained more figures than are significant because the internal consistency of the data is higher than their absolute accuracy. The maximum error of the data is $< \pm 0.1\%$.

We consider first the results for the conductances in pure H₂O and D₂O. By forming the ratio of the conductances at $X_D = 0$ and 1, we obtain an average factor $\Lambda(\text{HCl–H}_2\text{O})/\Lambda(\text{DCl–D}_2\text{O})$ of 1.364, which is independent of the acid concentration within the experimental precision. Frivold *et al.*¹⁵ report the value 1.362 for this factor, and other (presumably less accurate) data can be found in the compilation of Gmelin¹⁶. By extrapolation we obtain a limiting conductance Λ^0 of DCl in D₂O at 25 °C of 312.7 S cm² mol⁻¹, which, subtracting the limiting conductance of 62.83 S cm² mol⁻¹ for Cl⁻ in D₂O, yields a limiting conductance of the D⁺ ion in D₂O of 249.9 S cm² mol⁻¹. This compares well with the value for a mixture containing 99.35% D₂O, 250 S cm² mol⁻¹ (ref. 17).

Next we consider the results for H₂O–D₂O mixtures. For reasons discussed below, we define the deviation of the measured conductance of a mixture, $\Lambda(X_D, C)$, from that determined by linear interpolation between the values of the two pure solutions ($X_D = 0$ and 1), $\Lambda_{\text{lin}}(X_D, C)$ as

$$\begin{aligned} \Delta\Lambda(X_D, C) &= \Lambda(X_D, C) - \Lambda_{\text{lin}}(X_D, C) \\ &= \Lambda(X_D, C) - [(1 - X_D)\Lambda(0, C) + X_D\Lambda(1, C)] \end{aligned} \quad (1)$$

and the corresponding relative deviation in per cent by

$$\Delta^{\text{rel}}(X_D, C) \equiv 100\Delta\Lambda(X_D, C)/\Lambda_{\text{lin}}(X_D, C) \quad (2)$$

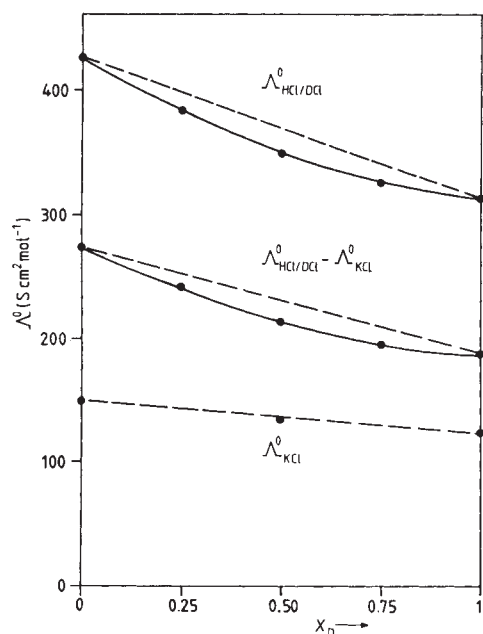


FIG. 1 Molar conductances of HCl–DCl and KCl in H₂O–D₂O mixtures at infinite dilution and $T = 25^\circ\text{C}$ as a function of the mole fraction X_D of deuterium. Also shown is the 'excess conductance', determined from the difference between the data for HCl–DCl and KCl. Solid and broken lines are guides to the eye. Error bars are smaller than the size of each data point.

TABLE 1 Molar conductances of HCl–DCl in H₂O–D₂O at 25 °C

C (mol dm ⁻³)	X_D				
	0	0.25	0.5	0.75	1
0.50	361.07	325.68	296.27	276.13	264.90
0.40	367.36	330.78	301.51	280.92	269.16
0.30	374.10	336.86	307.06	286.15	274.11
0.20	381.71	343.85	313.31	292.01	279.96
0.10	391.49	352.70	321.66	299.51	286.92
0.05	399.30	359.72	328.41	305.30	292.70
0.02	407.31	366.92	335.00	311.61	298.62
0.01	412.06	371.04	338.56	315.18	302.15
$C \rightarrow 0$	426.3	384.5	350.5	326.2	312.7
$\Delta\Lambda(X_D, C \rightarrow 0)$	—	-13.4	-19.0	-14.9	—
$\Delta^{\text{rel}}(X_D, C \rightarrow 0)$	—	-3.4%	-5.1%	-4.4%	—

Molar conductances Λ (S cm² mol⁻¹) of HCl–DCl in H₂O–D₂O mixtures at 25 °C as a function of solute–solvent composition (expressed by the atom fraction of deuterium, X_D) and of acid concentration C . $\Delta\Lambda(X_D, C \rightarrow 0)$ is the deviation of the molar conductance at infinite dilution of the acid from the value calculated by linear interpolation between the limiting conductances of the two pure solutions (with $X_D = 0$ and $X_D = 1$). $\Delta^{\text{rel}}(X_D, C \rightarrow 0)$ is the corresponding relative deviation in per cent. The maximum error of the data is $< \pm 0.1\%$.

The analysis of the experimental results shows that $\Delta^{\text{rel}}(X_D, C)$ is independent of the acid concentration, and we can therefore extrapolate to obtain the limiting conductances of HCl–DCl in H₂O–D₂O mixtures (Table 1). These results are in good agreement with the corresponding data at 0.05 mol dm⁻³ reported in ref. 17. Other data (see, for example, ref. 16) are also in qualitative agreement with our findings.

Figure 1 shows the resulting conductances at infinite dilution Λ^0 plotted against the mole fraction X_D . At intermediate solvent compositions the curve lies below the straight line connecting the values for pure H₂O and D₂O. The relative deviation at the equimolar solvent composition is $\sim -5.1\%$. The small experimental error of $\pm 0.1\%$ mentioned above strongly supports the accuracy of this result. The deviations $\Delta\Lambda(X_D, C \rightarrow 0)$ and $\Delta^{\text{rel}}(X_D, C \rightarrow 0)$ are given in Table 1. We emphasize that the relative decrease at the equimolar solvent composition, $\Delta^{\text{rel}}(0.5)$, is always about -5% , independent of the solute concentration C (Table 1).

The conductivity data for KCl in H₂O–D₂O mixtures are presented in Table 2 and Fig. 1. Our results are in good agreement with the conductance of KCl in pure H₂O and pure D₂O given in ref. 18. In an equimolar H₂O–D₂O mixture ($X_D = 0.5$) the observed deviation of the limiting conductance Λ^0_{KCl} from the corresponding linearly interpolated value is only slightly negative, about -0.8% . We note that there is also only a slight positive deviation (about $+0.95\%$) in the viscosity¹⁹ of this mixture and a small deviation in the self-diffusion coefficients²⁰.

We note that the excess conductance, defined as the difference between the data obtained for HCl–DCl and KCl, exhibits a corresponding relative decrease from linearity of -7.7% at $X_D = 0.5$. (This traditional procedure is based on the fact that many thermodynamic quantities of these two solutions are very similar¹⁴.)

The conductances of KCl solutions in H₂O–D₂O mixtures depend almost linearly on X_D which is as expected from standard electrochemical theory¹⁴ because it is well established¹⁹ that the fluidity (the inverse of the viscosity) of these mixtures depends almost linearly on X_D and ionic conductances are, to a very good approximation, direct proportional to the fluidity of the solvent (Walden's rule). Further, the linear dependence of Λ_{KCl} on X_D is independent of the concentration C . But our results show that there is a decrease of the molar conductance of HCl–DCl in the H₂O–D₂O mixtures from the expected linear dependence on X_D . This does not seem to be explicable by the standard theory (at least without the introduction of *ad hoc* assumptions). We are not aware of any classical theoretical treatment of this effect. Because this decrease occurs for HCl and DCl solutions but not for KCl solutions some property of H⁺ and D⁺ ions and H₂O–D₂O solvent must be involved.

TABLE 2 Molar conductances of KCl in H₂O–D₂O at 25 °C

C (mol dm ⁻³)	X _D		
	0	0.5	1
0.1	129.02	117.31	107.48
0.05	133.43	121.23	110.73
0.01	141.33	128.23	117.19
C → 0	150.0	136.06	124.3
ΔΛ(X _D , C → 0)	—	-1.09	—
Δ ^{rel} (X _D , C → 0)	—	-0.8%	—

See Table 1 notes for explanations.

This experiment was proposed⁹ as a critical test of a theoretical prediction concerning the possible existence of quantum correlations between H⁺ and protons of water molecules^{3,9}. This hypothesis was motivated by the detailed analysis of Hertz²¹ concerning the impossibility of directly deducing (from any known experiment) the existence of H⁺ as a well defined particle in aqueous solutions, and by the classic work of Eigen^{1,2} concerning the quantum tunnelling of protons in H₂O clusters.

In the framework of our general theory of quantum correlations⁸, the interpretation of the anomalous conductance is based on the following point⁹. If the well-known high H⁺ conductance, λ_{H⁺}, in liquid water is caused by the assumed specific quantum interference and delocalization effect (the coherent dissipative structures^{3,8}) then there must be an anomalous decrease of λ_{H⁺} in H₂O–D₂O mixtures owing to the mass and spin 'superselection rules'⁴. In these mixtures, the quantum interference between appropriate proton states becomes disrupted by deuterons 'belonging to' D₂O, HDO or D⁺ ions and being 'near' or 'between' the considered protons. This implies that the spatial extension of the corresponding coherent dissipative structures (which describe the delocalization of the protons participating in them) becomes restricted, causing a corresponding decrease of λ_{H⁺}. (The same conclusion holds for the D⁺ conductance; see, however, ref. 3). In general, quantum correlations have no classical analogue^{4-6,8}, so there are no 'molecular pictures' of these structures.

Our observations suggest that this effect may play an important part in the dynamics of proton transfer and hydrogen-bond formation²² in other physical, chemical and biological systems. Further work is in progress. □

Received 5 March; accepted 6 June 1990.

- Eigen, M. & de Maeyer, L. *Proc. R. Soc. A* **247**, 505–533 (1958).
- Eigen, M. *Angew. Chem.* **75**, 489–508 (1963); English translation, *Angew. Chem., int. Ed.* **3**, 1–19 (1964).
- Chatzidimitriou-Dreismann, C. A. & Brändas, E. J. *Int. J. Quantum Chem.* **37**, 155–165 (1990).
- Primas, H. *Chemistry, Quantum Mechanics and Reductionism* (Springer, Berlin, 1983).
- d'Espagnat, B. *Phys. Rep.* **110**, 201–264 (1984).
- d'Espagnat, B. *Conceptual Foundations of Quantum Mechanics* 2nd edn (Addison-Wesley, Redwood City, 1989).
- Einstein, A., Podolsky, B. & Rosen, N. *Phys. Rev.* **47**, 777–780 (1935).
- Brändas, E. J. & Chatzidimitriou-Dreismann, C. A. in *Resonances* (eds Brändas, E. & Elander, N.) Lecture Notes in Physics, Vol. 325, 485–540 (Springer, Berlin, 1989).
- Chatzidimitriou-Dreismann, C. A. *Int. J. Quantum Chem., Symp.* **23**, 153–158 (1989).
- Stokes, R. H. *J. phys. Chem.* **65**, 1242–1247 (1961).
- Haase, R., Sauerman, P. F. & Dücker, K. H. *Z. phys. Chem.* **47**, 224–245 (1965).
- Kell, G. S. *J. chem. Ref. Data* **6**, 1109 (1977).
- Longworth, L. G. *J. phys. Chem.* **64**, 1914–1917 (1960).
- Robinson, R. A. & Stokes, R. H. *Electrolyte Solutions* (Butterworths, London, 1970).
- Frivold, O. E., Hassel, O. & Hetland, E. *Avhandl. Norske Videnskaps Akad. Oslo I Mat. Naturv. Kl.* No. 9, 1 (1942).
- Gmelin's *Handbuch der Anorganischen Chemie*, Chlor, Suppl. Issue Part B1, 8 edn (Verlag-Chemie, Weinheim, 1969).
- Longworth, L. G. & McInnes, D. A. *J. Am. chem. Soc.* **59**, 1666–1670 (1937).
- Swain, D. G. & Evans, D. F. *J. Am. chem. Soc.* **88**, 383–390 (1966).
- Jones, G. & Fornwald, H. *J. chem. Phys.* **4**, 30–33 (1936).
- Weingärtner, H. *Ber. Bunsenges. phys. Chem.* **88**, 47–50 (1984).
- Hertz, H. G. *Chem. Scripta* **27**, 479–493 (1987).
- Zundel, G. & Eckert, M. *J. molec. Struct.* **200**, 73–92 (1989).

ACKNOWLEDGEMENTS. We thank G. Ertl for discussions and for suggesting this experiment, E. Brändas and G. Hertz for discussions, and U. Schneider for assistance in some of the measurements. This work was supported by the Commission of the European Communities, the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie.

High-resolution ²⁷Al NMR spectroscopy of the aluminophosphate molecular sieve VPI-5

Y. Wu*, B. F. Chmelka*, A. Pines*§, M. E. Davis†, P. J. Grobet‡ & P. A. Jacobs‡

* Materials and Chemical Sciences Division, Lawrence Berkeley Laboratory, 1 Cyclotron Road, Berkeley, California 94720 and Department of Chemistry, University of California, Berkeley, California 94720, USA

† Department of Chemical Engineering, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061, USA

‡ Laboratorium voor Oppervlaktechemie, Catholic University of Leuven, B-3030 Leuven, Belgium

ALUMINIUM plays an important part in determining the properties of many materials, such as the catalytic behaviour of zeolites. Aluminophosphate molecular sieves, in particular, have useful applications as superlattice hosts in the fabrication of quantum-effect devices¹. Although nuclear magnetic resonance (NMR) spectroscopy is often a sensitive probe of solids, the use of ²⁷Al NMR to investigate the structure of aluminosilicates and aluminophosphates has been severely limited because anisotropic second-order quadrupolar interactions, responsible for spectral broadening, cannot be eliminated by conventional magic-angle-spinning or multiple-pulse techniques. Here we report the first high-resolution NMR spectra of ²⁷Al in a solid using double rotation²⁻⁴ and demonstrate its usefulness for probing subtle structural perturbations in the aluminophosphate molecular sieve VPI-5^{5,6}. From our results, we conclude that high-resolution ²⁷Al NMR is capable of resolving discrete framework aluminium sites, permitting quantitative investigation of site-specific adsorbate interactions with the VPI-5 host.

VPI-5 is a hydrophilic molecular sieve belonging to the aluminophosphate (AlPO₄) family. The proposed structure of VPI-5 in Fig. 1, based on X-ray and neutron powder diffraction experiments⁵⁻⁸, consists of alternating AlO₄ and PO₄ tetrahedral units arranged to produce linear channels 12 Å in diameter. There are two crystallographically distinct aluminium sites: one located between the two four-membered rings (Al-1 in Fig. 1) and the other situated in the six-membered rings (Al-2). The ratio of the number of Al-1 to Al-2 sites is 1:2, and the hexagonal unit-cell parameters *a* and *c* are 19.009 Å and 8.122 Å, respectively, in the hydrated material. On dehydration, structural changes result in a contraction of *a* to 18.549 Å and an expansion of *c* to 8.404 Å (ref. 7). Such local perturbations in the framework lead to changes in chemical shifts and quadrupole interactions of the ²⁷Al nucleus. In a resolved NMR spectrum the isotropic chemical shift δ, the quadrupole-coupling constant ν_Q and the asymmetry parameter η should provide information about the symmetry and structure at the aluminium sites in the aluminophosphate framework.

We carried out ²⁷Al NMR experiments in a pulsed spectrometer operating at 104.23 MHz using a standard magic-angle-spinning (MAS) probe as well as a newly developed double-rotation (DOR) probe⁹. Figure 2a shows the ²⁷Al MAS spectrum of hydrated, polycrystalline VPI-5 spinning at 5 kHz. As observed previously^{8,10}, it contains an asymmetric peak at ~41 p.p.m. and a second peak below 0 p.p.m. The resonance at 41 p.p.m. falls in the range of isotropic shifts attributed to tetrahedrally coordinated Al sites, Al(IV), whereas the peak below 0 p.p.m. is attributed to octahedrally coordinated Al sites, Al(VI) (ref. 11). Figure 2b shows the DOR spectrum of the same hydrated sample of VPI-5. Two peaks, a and b, of equal

§ To whom correspondence should be addressed.

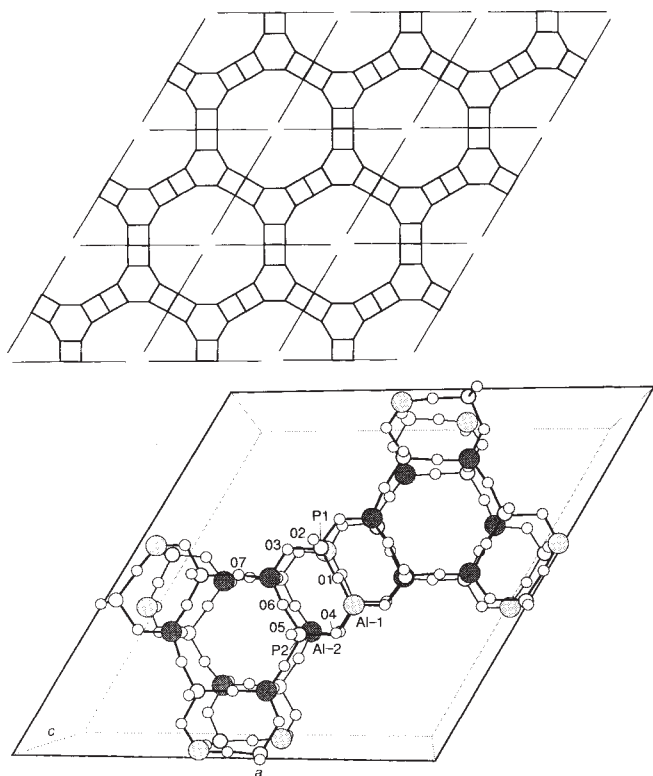


FIG. 1 The VPI-5 framework structure. Top, [001] projection, where the black lines represent oxygen atoms bonded to aluminium and phosphorus atoms at the vertices. Bottom, Hexagonal unit cell with labelled atoms.

intensity and with 1-p.p.m. linewidths at 41.2 and 40.4 p.p.m., are partially resolved, as well as a single peak, c, at -18.4 p.p.m. The intensities of the peaks are in a 1:1:1 ratio, so peak c at -18.4 p.p.m. apparently originates from half of the Al-2 sites which chemisorb water to acquire octahedral coordination as Al-2(VI). The remaining Al-2(IV) sites and all of the Al-1(IV) sites produce the two partially resolved peaks near 41 p.p.m. Peaks a and b have quite different quadrupole interaction param-

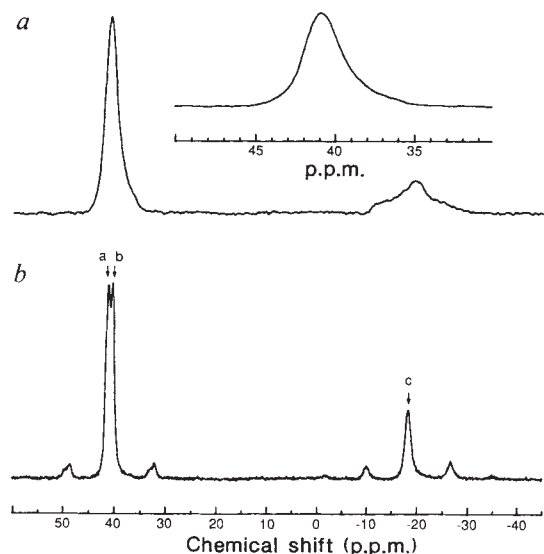


FIG. 2 ^{27}Al NMR spectra of hydrated VPI-5 under conditions of magic-angle spinning (a) and double rotation (b). Peaks not indicated by arrows are spinning sidebands. Isotropic DOR peak positions reflect contributions from both the isotropic chemical shift and the isotropic second-order quadrupole shift. Spectra are referenced to dilute aqueous $\text{Al}(\text{NO}_3)_3$.

eters (Y.W. *et al.*, manuscript in preparation), consistent with their assignment to the different framework aluminium positions. One cannot, however, rule out the possibility that Al-1 sites become octahedrally coordinated during hydration, distorting the lattice and rendering the Al-2 sites inequivalent. VPI-5 can contain up to ~24 wt% water ($\text{AlPO}_4 \cdot 2.3\text{H}_2\text{O}$) in the fully hydrated state, indicating that both chemisorption ($\text{Al(IV)} \rightarrow \text{Al(VI)}: \text{AlPO}_4 \cdot 0.67\text{H}_2\text{O}$) and physisorption of water occur.

Figure 3a, b shows the ^{27}Al MAS spectrum for VPI-5 spinning at 5 kHz and the ^{27}Al DOR spectrum of the same sample, both acquired immediately after room-temperature dehydration for 48 h at 10^{-5} torr. Two peaks unresolved in the MAS spectrum, d at 33.3 p.p.m. and e at 35.9 p.p.m., are observed in the DOR spectrum. From the 1:2 intensity ratio of these two peaks, peak d is assigned to Al-1 sites and peak e is attributed to Al-2 sites, both of which represent tetrahedrally coordinated aluminium. During dehydration, Al(VI) sites are converted into Al(IV) sites, consistent with the disappearance of peak c at -18.4 p.p.m. Furthermore, in hydrated VPI-5, ^{27}Al tetrahedral sites (peaks a and b) are altered by dehydration to yield different ^{27}Al tetrahedral environments (peaks d and e). The weak peak f at 22.8 p.p.m. may arise from partial hydration of Al sites coordinated

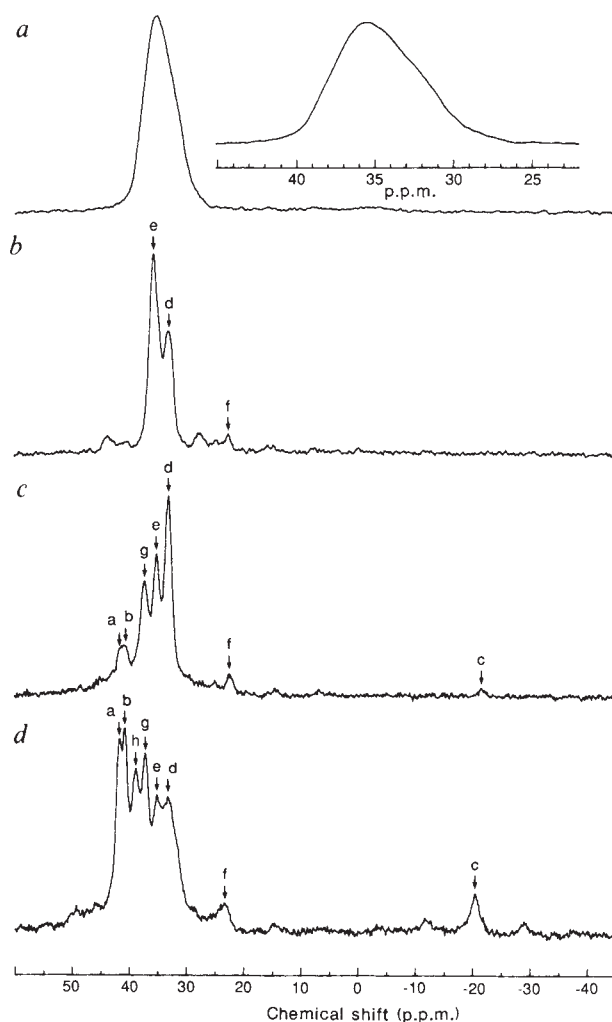


FIG. 3 ^{27}Al NMR spectra of dehydrated and partially rehydrated VPI-5. a, MAS spectrum of dehydrated VPI-5. b, DOR spectrum of dehydrated VPI-5. c, DOR spectrum after two days of rehydration. d, DOR spectrum after 23 days of rehydration. Spectra are referenced to dilute aqueous $\text{Al}(\text{NO}_3)_3$ and are plotted on arbitrary intensity scales.

to one water molecule. Figure 3c, d shows ^{27}Al DOR spectra taken 2 days and 23 days after dehydration; the sample remained inside the inner rotor of the DOR probe during this period, allowing water molecules to diffuse slowly into the material. The two sharp lines that emerge, g at 37.3 p.p.m. and h at 38.8 p.p.m., arise from Al-2 species influenced by adsorbed water molecules. Figure 3c shows that the relative intensity of peak d associated with dehydrated Al-1 sites undergoes little change as a result of partial rehydration, bearing in mind that the 1:2 ratio of Al-1 to Al-2 species must be maintained at all stages of hydration. The sensitivity of the VPI-5 framework to guest loading and packing, as indicated by these data, should yield new insight into the behaviour of molecules in confined micro-porous environments.

Both DOR and dynamic angle spinning (DAS) (recently demonstrated for ^{17}O and ^{23}Na (ref. 12)) make it possible to measure ^{27}Al quadrupole-coupling parameters and chemical shifts, allowing the study of adsorbate-host interactions in molecular sieves and subtle microstructural features in other aluminium-containing solids including catalysts, glasses, ceramics, minerals and semiconductors. $\square$

Received 3 April; accepted 4 June 1990.

1. Stucky, G. D. & MacDougall, J. E. *Science* **247**, 669–678 (1990).
2. Llor, A. & Virlet, J. *Chem. Phys. Lett.* **152**, 248–253 (1988).
3. Samoson, A., Lippmaa, E. & Pines, A. *Mol. Phys.* **65**, 1013–1018 (1988).
4. Chmelka, B. F. et al. *Nature* **339**, 42–43 (1989).
5. Davis, M. E., Saldarriaga, C., Garces, J. M. & Crowder, C. *Nature* **331**, 698–699 (1988).
6. Crowder, C. E., Garces, J. M. & Davis, M. E. *Adv. X-ray Analysis* **32**, 503–510 (1989).
7. Richardson, J. W. Jr, Smith, J. V. & Pluth, J. J. *J. phys. Chem.* **93**, 8212–8219 (1989).
8. Davis, M. E. et al. *J. Am. chem. Soc.* **111**, 3919–3924 (1989).
9. Wu, Y., Sun, B. Q., Pines, A., Samoson, A. & Lippmaa, E. *J. magn. Res.* (in the press).
10. Grobet, P. J. et al. *Appl. Catal.* **56**, L21–L27 (1989).
11. Blackwell, C. S. & Patton R. L. *J. phys. Chem.* **88**, 6135–6139 (1984).
12. Mueller, K. T. et al. *J. magn. Res.* **86**, 470–487 (1990).

ACKNOWLEDGEMENTS. This work was supported by the Office of Basic Energy Sciences, Materials and Chemical Sciences Division, US Department of Energy and by the Shell Foundation. The authors thank J. A. Martens for assistance with VPI-5 sample preparation. B.F.C. is a NSF post-doctoral fellow.

Importance of biomass burning in the atmospheric budgets of nitrogen-containing gases

Jürgen M. Lobert, Dieter H. Scharffe, Wei M. Hao & Paul J. Crutzen

Department of Atmospheric Chemistry, Max-Planck-Institut für Chemie, PO Box 3060, 6500 Mainz, FRG

BIOMASS burning is a primary source of many trace substances that are important in atmospheric chemistry^{1–6}. More than 80% of the world's biomass burning takes place in the tropics³ as a result of savanna fires, forest-clearing activity, and the burning of agricultural waste and wood. Here we report results from laboratory studies on the emission of nitrogen-containing compounds from the burning of dry vegetation. We find that the emission rates of NO_x , HCN and CH_3CN are sufficient to contribute significantly to the global atmospheric budget of the compounds. Furthermore, possibly up to half of the biomass nitrogen can be converted to molecular nitrogen, N_2 , leading to an estimated annual loss of $12\text{--}28 \times 10^{12}$ g of biomass nitrogen ('pyrodenitrification'), equal to $\sim 9\text{--}20\%$ of the estimated global rate of terrestrial nitrogen fixation.

According to recent estimates³, $\sim 3\text{--}6 \times 10^{15}$ g of biomass carbon is burned annually, corresponding to $24\text{--}57 \times 10^{12}$ g of biomass nitrogen. This burning produces mostly CO_2 , but also 10% CO and $\sim 2\%$ CH_4 and other hydrocarbons. Many other gases and particulate matter are also emitted. Here we consider the emissions of nitrogen-containing compounds.

Our results have been obtained using a small-scale burning apparatus built to simulate open fires (Fig. 1). The details of this apparatus are described elsewhere⁶. Because most of the biomass burning takes place in the tropics, we burned mostly tropical grasses from savanna regions and also agricultural wastes, in a total of 41 burning experiments.

Our analytical system was designed to determine CO_2 , CO, non-methane hydrocarbons (NMHC) and CH_4 as well as the most important nitrogen-containing species: NO_x (NO and NO_2), ammonia (NH_3), some cyanogen compounds such as hydrogen cyanide (HCN) and acetonitrile (CH_3CN), and nitrous oxide (N_2O). Determination of the flow rate in the stack, the weight loss of the fuel, and the elemental content of both biomass and ash, as well as the concentrations of gaseous emissions, enabled us to carry out a mass balance for each experiment.

Our apparatus also allows us to observe the different burning stages separately. A burn can be divided into a hot flaming phase emitting oxidized compounds such as CO_2 , NO_x and N_2O , and a colder, incompletely combusting smouldering phase producing much more smoke and less oxidized substances such as CO, hydrocarbons, ammonia and nitriles⁶. Figure 2 shows some of the compounds emitted during one of our experiments, and the corresponding stack-gas temperature. The transition between flaming and smouldering combustion is clearly observable in our experiments and corresponds to a large increase in the CO concentration and a decrease in CO_2 emission at ~ 96 s.

Our data show that on average $\sim 90\%$ of the biomass nitrogen and 95% of the carbon were volatilized during the burn, with a mean weight loss of 75% in the flaming and 25% in the smouldering stage. Almost all of the carbon was recovered in the measured emissions of CO_2 , CO, hydrocarbons and in the ash. By contrast, only $\sim 32\%$ of the nitrogen could be regained by the above mentioned nitrogen-containing compounds and the nitrogen content of the ash (Table 1).

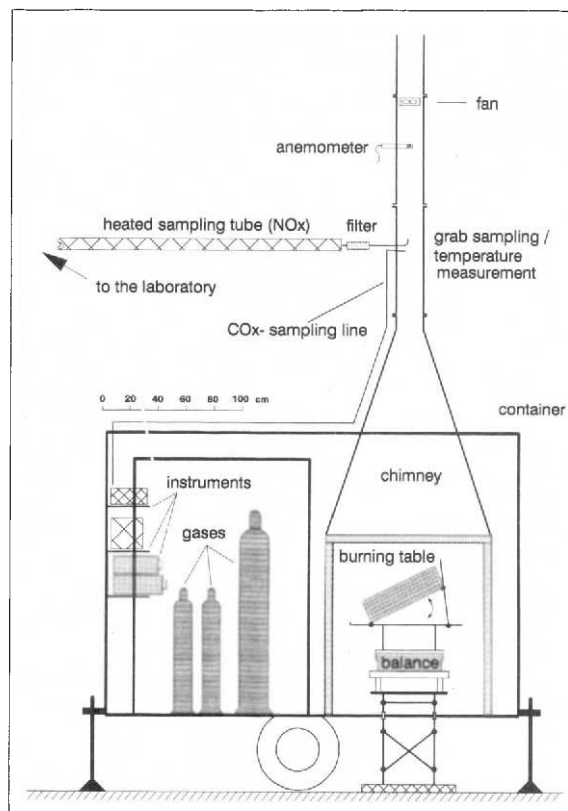


FIG. 1 Burning apparatus for an experimental simulation of open biomass burning⁶.

TABLE 1 Nitrogen balance of burning experiments

Compound	Percentage of biomass nitrogen	Number of experiments	Source* (10^{12} g N yr $^{-1}$)	Global† (10^{12} g N yr $^{-1}$)	Ref.
NO $_x$	12.7 $\pm$ 4.94	22	2.46–8.65	25–99	11
NH $_3$	4.00 $\pm$ 3.05	15	0.59–3.15	20–80	4
HCN	2.42 $\pm$ 1.79	12	0.37–1.89	?	
CH $_3$ CN	0.95 $\pm$ 0.74	11	0.14–0.75	?	
Other nitriles	0.21 $\pm$ 0.21	10	0.03–0.18	?	
N $_2$ O	0.77 $\pm$ 0.21	12	0.16–0.50	14	12
Amines ⁷	0.24 $\pm$ 0.21	5	0.03–0.20	?	
HNO $_3$	$\approx$ 1	2	0.24–0.57	?	
Residue (ash)	9.89 $\pm$ 0.95	41	2.26–5.91		
Sum	32.2				
Unknown	67.8				
Higher N-compounds	20?		5–11		9
N $_2$	50?		12–28	139‡	10

* Annual source strength for the compounds using our emission ratios and the global biomass-burning nitrogen volatilization of $24\text{--}57 \times 10^{12}$ g N yr $^{-1}$ (ref. 3). In estimating the final range we multiplied the mean value $\pm$ half of the standard deviation with the range of nitrogen release. For example, source of NO $_x$ = $(12.7 \pm 2.47) \times (24\text{--}57)$.

† Global budgets of the gases and reference for these estimates.

‡ Global terrestrial nitrogen fixation rate.

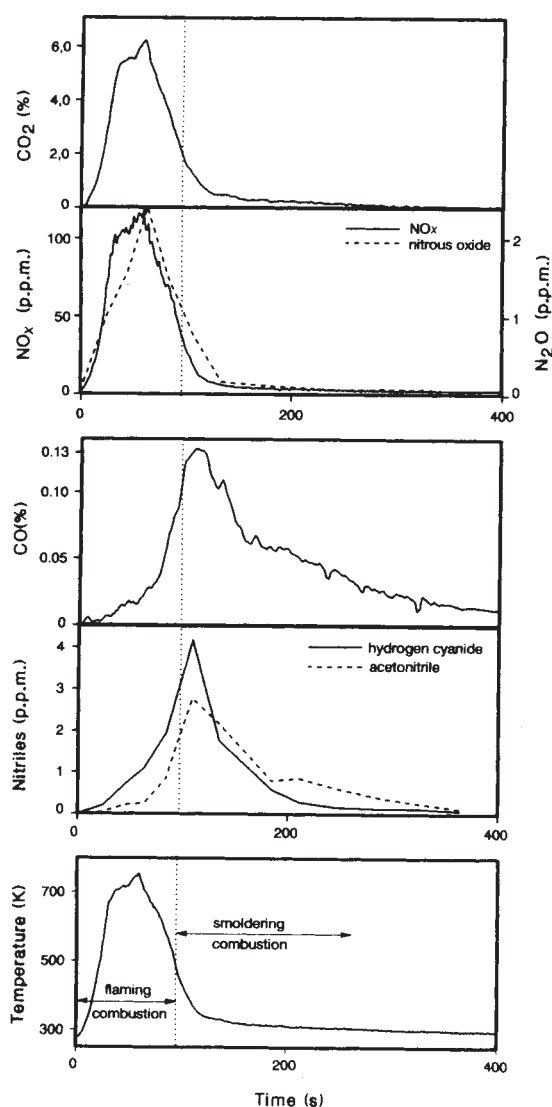


FIG. 2 Mixing ratios of some gases in the fire exhaust, and the stack-gas temperature of a burning experiment, as a function of time. The dotted vertical line represents the transition between flaming and smoldering stages⁶.

The most important emissions in terms of the original nitrogen content of the fuel were NO $_x$ ($\sim$ 13%), ammonia ($\sim$ 4%), HCN (2.4%) and acetonitrile ($\sim$ 1%). Minor contributions were made by N $_2$ O, other nitriles, C $_1$ to C $_5$ amines⁷ and other nitrogen oxides (mainly HNO $_3$).

The NO $_x$ emissions from tropical biomass burning account for $\sim$ 20% of the global budget¹¹ whereas ammonia emissions could represent $\sim$ 10% of the global source⁴. What is remarkable is the high amount of nitriles emitted during biomass burning. These emissions may dominate the atmospheric inputs of hydrogen cyanide and acetonitrile, being much greater than automotive and industrial emissions. Therefore, HCN and CH $_3$ CN may well serve as global tracers for biomass burning emissions. The most important atmospheric sink for these gases, the reaction with OH $^{\cdot}$ radicals (which consumes about 0.17×10^{12} and 0.40×10^{12} N yr $^{-1}$ for HCN and CH $_3$ CN respectively), is much smaller than their biomass-burning source, implying that other important sinks must exist; these probably include uptake in the ocean⁸ and in plants⁶. Our results also show that biomass burning does not contribute significantly to the global budget of nitrous oxide.

Our measurements do not include all organic nitrogen compounds produced by incomplete combustion. As an indication of their relative importance, we may consider the product distribution in tobacco smoke, which contains mainly aliphatic and aromatic amines and heterocyclic constituents as well as the compounds considered here⁹. Using this distribution we estimate that such compounds can account for only another 20% of the plant nitrogen.

This implies that up to 50% of the original fuel nitrogen was emitted in a form that neither was detected during our own experiments nor can be deduced from analyses of tobacco effluents. Thus molecular nitrogen, which cannot be measured in open fires, is a likely candidate for the missing nitrogen. Some preliminary burning experiments in a closed apparatus supplied with a helium/oxygen mixture indeed show that a significant fraction of N $_2$ is formed (T. A. Kuhlbusch, personal communication). If the missing 50% does indeed consist of N $_2$, this represents an annual loss of $12\text{--}28 \times 10^{12}$ g N from tropical ecosystems. Such a rate of loss in plant nitrogen equals $\sim$ 9–20% of the estimated annual, global, terrestrial nitrogen fixation rate (139×10^{12} g N yr $^{-1}$)¹⁰ and an even higher proportion from tropical ecosystems, especially savannas and agricultural lands.

Thus biomass burning is important not only to atmospheric chemistry but, because of nitrogen gas release and pyrodenitrification, also to the biogeochemical nitrogen cycle of tropical savannas and agricultural ecosystems. Because most

biomass burning takes place as a result of human activity³, its long-term effect on the functioning of these ecosystems is an important issue that warrants further exploration. □

Received 23 May; accepted 26 June 1990.

1. Crutzen, P. J., Heidt, L. E., Krasnek, J. P., Pollock, W. H. & Seiler, W. *Nature* **282**, 253–256 (1979).
2. Crutzen, P. J. *et al.* *J. Atmos. Chem.* **2**, 233–256 (1985).
3. Crutzen, P. J. & Andreae, M. O. *Science* (in the press).
4. Andreae, M. O. *et al.* *J. geophys. Res.* **93**, 1509–1527 (1988).
5. Cofer, W. R. *J. geophys. Res.* **94**, 2255–2259 (1989).
6. Lobert, J. M. thesis, Max-Planck-Institut für Chemie (Mainz) (1990).
7. Seuwen, R. thesis, Max-Planck-Institut für Chemie (Mainz) (1989).
8. Hamm, S. & Warneck, P. *J. geophys. Res.* (in the press).
9. Neurath, G. *Beitr. Tabakforsch.* **5**, 115–133 (1969).
10. Soderlund, R. & Svensson, B. H. *Ecol. Bull., Stockholm* **22**, 33–73 (1976).
11. Logan, J. *J. geophys. Res.* **88**, 10785–10807 (1983).
12. World Health Organization *Global Ozone Research and Monitoring Proj.* Rep. 16 (WHO, Geneva, 1985).

ACKNOWLEDGEMENTS. We thank F. Flocke for permission to use unpublished results of HNO_3 measurements, and W. Dindorf and coworkers for elemental analyses.

An ice-core record of atmospheric response to anthropogenic sulphate and nitrate

P. A. Mayewski, W. B. Lyons, M. J. Spencer, M. S. Twickler, C. F. Buck & S. Whitlow

Glacier Research Group, Institute for the Study of Earth, Oceans and Space, University of New Hampshire, Durham, New Hampshire 03824, USA

RECORDS of sulphate and nitrate concentrations in ice cores show that these concentrations have increased recently because of the long-range transport of pollution from middle latitudes^{1–5}. But these records have been neither complete enough nor long enough to allow an assessment of their sensitivity to variations in the emissions of sulphate and nitrate precursors. We have now analysed sections from an ice core in South Greenland which have allowed us to extend its sulphate and nitrate record back from 1869 to 1767. This longer record has enabled us to determine the pre-industrial natural interannual variability of non-sea-salt sulphate and nitrate. We find that the background concentration in the remote atmosphere over South Greenland is sensitive to changes in the anthropogenic emissions of sulphate and nitrate, and responds to these variations on a timescale of the order of decades.

The highest resolution, continuous glaciochemical time-series available for Greenland¹ (site 20D; located 40 km southwest of

Dye 3 to avoid any local pollution effects⁶) previously covered the period 1869–1984. Analysis of the remainder of the core has now provided a detailed record extending back to 1767 (Fig. 1).

For south Greenland the most probable sources of sulphate are: sea-salt aerosol (for which Fig. 1 has been corrected) oxidation of the dimethylsulphide (DMS) produced by phytoplankton and emitted from the oceans; natural stratospheric aerosols; volcanic emissions via SO_2 ; and anthropogenic emissions via SO_2 (primarily stationary-source fuel combustion⁷).

The principal source of nitrate to the site is anthropogenic activity (mainly stationary-source fuel combustion and transportation-related emissions⁷ in addition to biomass burning). Other sources include lightning, NH_3 oxidation and NO exhalation⁸. Biomass burning is probably a more effective source of NO_x for the Southern Hemisphere free troposphere.

Probability plots (Fig. 2) verify the major trends indicated in the extended time-series for non-sea-salt (n.s.s.) sulphate and nitrate (Fig. 1). For n.s.s. sulphate the following time periods can be differentiated: 1767–1903, 1903–1960 and 1960–1984 during which the ranges in concentration are 10–154, 28–77 and 41–93 ng g^{-1} , respectively. An estimate of the concentration range in n.s.s. sulphate, corrected for volcanic input (from identifiable discrete events and not including volcanic background) can be determined from the probability plots by subtracting the number of years affected by volcanic activity per period⁹ from the upper end of the probability plot (8% for 1960–84, 31% for 1903–60; 25% for 1767–1903, estimated from the total 1869–1984 record). The concentration ranges for non-volcanic n.s.s. sulphate then become 10–28, 28–51 and 41–84 ng g^{-1} .

Measurements of methanesulphonic acid, an atmospheric oxidation product of dimethylsulphide (DMS), provide an estimate of the general strength of the biogenic sulphur signal at site 20D (decadal mean values $< 4.5 \text{ ng g}^{-1}$ and highs $< 10 \text{ ng g}^{-1}$; E. Saltzman, personal communication). Thus, for the period 1767–1903 two-thirds or more of the n.s.s. non-volcanic (discrete

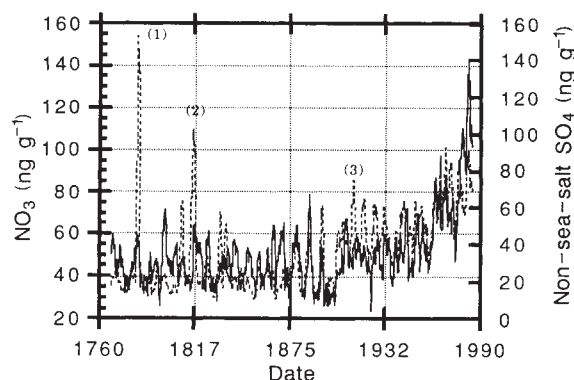


FIG. 1 Time-series of the non-sea-salt sulphate (dashed line) and nitrate (solid line) concentrations at site 20D. Data have been smoothed (using a gaussian function) to 1–2 years from the original ~ 5.8 samples per year, to remove seasonal signals not discussed here. Examples of volcanic events recorded by n.s.s. sulphate spikes are: (1) Laki (1783); (2) Tambora (1815); (3) Katmai (1912).

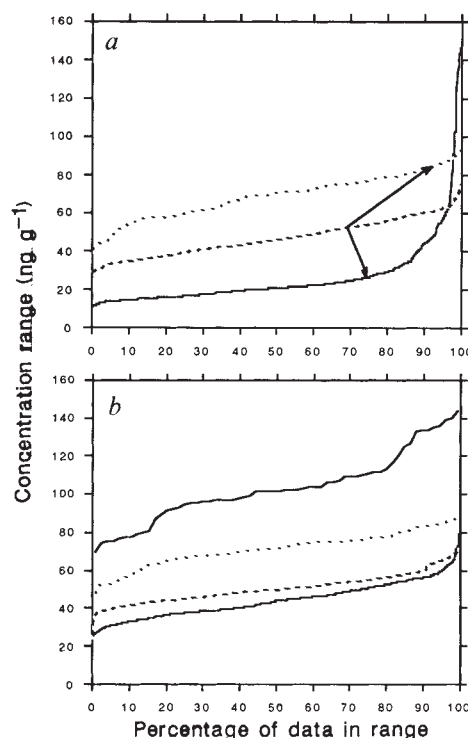


FIG. 2 *a*, Probability plot for n.s.s. sulphate (solid line, 1767–1903; dashed line, 1903–1960; dotted line, 1960–1984). *b*, Probability plot of nitrate (upper solid line, 1767–1903; dotted line, 1960–1976; dashed line, 1903–1960; lower solid line, 1976–1984). Both plots were produced using a normalized linear probability (percentage) distribution.

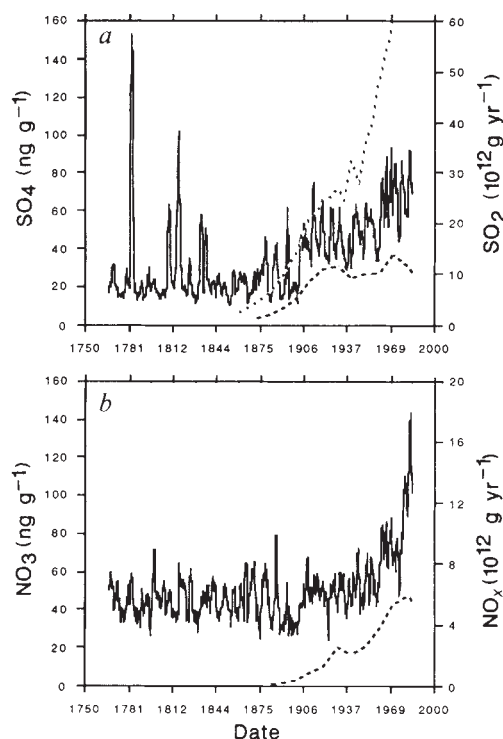


FIG. 3 *a*, Concentration of n.s.s. sulphate (solid line) compared with the anthropogenic global SO_2 (ref. 10) (dotted line) and US SO_2 (ref. 13) (dashed line) emissions. *b*, Concentration of nitrate (solid line) compared with the anthropogenic US NO_x (ref. 13) emission.

event) sulphate deposited in south Greenland was derived from natural stratospheric sulphate aerosols.

For the portion of the record 1767–1903 most of the large perturbations are believed to be associated with volcanic events⁹ and there is no apparent trend in n.s.s. sulphate (Fig. 1). Because the record extends back before the onset of significant industrialization we can calculate both a mean n.s.s. sulphate and a mean non-volcanic (discrete event) n.s.s. sulphate of ~ 20 and $\sim 18 \text{ ng g}^{-1}$, respectively, for pre-anthropogenic deposition (during the eighteenth and nineteenth centuries) in this region.

Except for the dramatic volcanic events of the period 1767–1903, n.s.s. sulphate concentrations for 1903–1960 fall outside the concentration range of the former period, and those for 1960–1984 fall outside the 1903–1960 range. The mean n.s.s. sulphate and mean non-volcanic n.s.s. sulphate for 1903–1960 are ~ 45 and $\sim 42 \text{ ng g}^{-1}$, respectively. For the period 1960–1984 mean n.s.s. and mean non-volcanic n.s.s. are ~ 70 and $\sim 69 \text{ ng g}^{-1}$, respectively.

Although volcanic events stand out in the record they do not seem to influence significantly the mean concentrations. As the marked increases in non-volcanic n.s.s. sulphate at this site also do not seem to be related to oxidation of DMS, the only likely source is oxidation of pollutant SO_2 .

Four time periods are distinguishable from the nitrate time-series (Fig. 1) using the probability plots (Fig. 2): 1767–1903, 1903–1960, 1960–1976 and 1976–1984 with concentration ranges of 24–73, 35–80, 48–87 and 69–144 ng g^{-1} , respectively. The pre-anthropogenic period 1767–1903 contains intriguing maxima and minima, but no apparent trend (Fig. 1).

Although the $\sim 50\%$ rise in the low end of the range for the period 1903–1960 falls within the overall range for snow derived from the natural atmosphere at this site, year-to-year variability during this period is reduced compared with the previous time period and there is a slight overall increase in concentration (Fig. 2). As there is no documented evidence of dramatic changes in lightning, NH_3 oxidation or NO -exhalation source strengths, we suggest that the rise is due to the addition of anthro-

pogenically derived nitrate. Therefore both n.s.s. sulphate and nitrate records from 1903 to the present reflect inputs from anthropogenic sources, which were appreciable by this time. Previous results suggested that NO_x emissions were not detectable in south Greenland until the 1950s to 1960s^{1,2} but our results show that by 1960 background concentrations were double those in 1767–1903.

Air masses entering south Greenland reflect variable inputs from sources in both North America and Eurasia. Because these regions contribute significantly to global anthropogenic production of SO_2 ^{10,11} and of NO_x ^{11,12} it is reasonable to compare the record with estimates of anthropogenic emissions of SO_2 and NO_x (Fig. 3). Although no time-series of global anthropogenic emissions of NO_x are available for comparison with the nitrate record, such estimates are available for SO_2 ¹⁰. To assess the effect of one of the major source areas for south Greenland, we also examined time-series of SO_2 and NO_x emission estimates available for United States¹³ (Fig. 3).

Background concentrations (identified by minima in the record) of n.s.s. sulphate parallel closely both global and US emission estimates until ~ 1940 with perhaps the exception of the abrupt rise in n.s.s. sulphate at ~ 1903 . We suggest that a coincidental period of pronounced volcanic activity over the period ~ 1900 –1910^{1,4} in combination with one of the sharpest rises in SO_2 emissions in the record may explain this dramatic jump. Because pre-1940 global and US emission trends are very similar it is not possible to determine which source dominated during this period. For the period ~ 1940 –1980 the trend in background concentrations is most similar to that of US emission estimates suggesting a primarily US source. Although Arctic haze may contribute to the pollutant sulphate burden in south Greenland, the transport pathways¹⁴ for this source make it an unlikely major contributor.

Background nitrate concentrations at site 20D and US emission estimates are parallel from ~ 1930 –1980 (Fig. 3). However, if the emission estimate is correct, it does not explain the rapid rise in nitrate at 20D starting at ~ 1903 . Furthermore, the fairly level background values ~ 1903 –1930 do not seem to be simply related to increases in NO_x emissions.

It is perhaps not surprising that background concentrations in n.s.s. sulphate follow more closely the trend in one source region and that the situation is more complicated for nitrate. Nitrate deposited at this site may be expected to originate from a broader source area than sulphate because much of the nitrate precursor is transported as a gas whereas much of the sulphate is associated with particles.

Although the trends of background concentrations and emission estimates are similar there is not enough year-to-year variability in the emission estimates to explain the variability in either the n.s.s. sulphate or nitrate records. Because this variability is not unique to the anthropogenic portion of the record we suggest that this variability represents the effect of year-to-year differences in circulation affecting this region. The lack of consistent correspondence between timing of maxima and minima of n.s.s. sulphate and nitrate is further evidence of differences in their mode of transport. Future investigations of the patterns in variability of these two species in ice cores could yield valuable information related to changes in atmospheric circulation.

We conclude that the anthropogenic emissions have significantly affected the sulphur and nitrogen cycles of the remote atmosphere in the Northern Hemisphere. Although year-to-year variability may be largely dominated by variability in atmospheric circulation, we suggest that background concentrations in the remote atmosphere maintain a signature that responds to, at the very least, patterns in the strength of pollutant sources on the order of decades or less. Although pre-anthropogenic concentrations of nitrate exceeded those of sulphate (Fig. 1), at the turn of the nineteenth century n.s.s. sulphate levels caught up to nitrate levels in response to dramatic large increases in the emissions of pollutant SO_2 (Fig. 3). By the mid-1970s,

however, nitrate concentrations in south Greenland again exceeded n.s.s. sulphate concentrations reflecting the more rapid rise of NO_x pollutant emissions (Fig. 3).

As both H_2SO_4 (by radiation shielding) and odd-N compounds (by the photochemistry of atmospheric O_3) have important roles in determining the temperature of the atmosphere, models dealing with global warming should include the effect of the marked increases in both SO_2 and NO_x for periods just after the turn of the nineteenth century. $\square$

Received 27 February; accepted 29 May 1990.

1. Mayewski, P. A. *et al. Science* **232**, 975–977 (1986).
2. Neftel, A., Beer, J., Oeschger, H., Zurcher, F. & Finkel, R. C. *Nature* **314**, 611–613 (1985).
3. Finkel, R. C. & Langway, C. C. Jr. *J. geophys. Res.* **91**, 9849–9855 (1986).
4. Steffensen, J. P. *Ann. Glaciol.* **10**, 171–177 (1988).
5. Wagenbach, D., Munnich, K. O., Schotterer, U. & Oeschger, H. *Ann. Glaciol.* **10**, 183–187 (1988).
6. Mayewski, P. A., Spencer, M. J., Lyons, W. B. & Twickler, M. S. *Atmos. Envir.* **21**, 863–869 (1987).
7. *National Air Pollutant Emission Estimates, 1940–1987, EPA-4014-58-022* (U.S. EPA Office of Air, Noise and Radiation, Office of Air Quality Planning and Standards Research, Triangle Park, 1989).
8. Legrand, M. R. & Kirchner, S. *J. geophys. Res.* **95**, 3493–3509 (1990).
9. Lyons, W. B., Mayewski, P. A., Spencer, M. J. & Twickler, M. S. *Ann. Glaciol.* (in the press).
10. Moller, D. *Atmos. Envir.* **18**, 19–27 (1984).
11. Hameed, S. & Dignon, J. *Atmos. Envir.* **22**, 441–449 (1988).
12. Logan, J. *J. geophys. Res.* **88**, 10785–10807 (1983).
13. Galloway, J. N. *Ambio* **18**, 161–166 (1989).
14. Raatz, W. E. & Shaw, G. E. *J. Clim. appl. Met.* **23**, 1052–1064 (1984).

ACKNOWLEDGEMENTS. We thank D. Barrett, J. James, C. Wake and K. Welch for their assistance, R. Delmas, J. Dibb, M. Legrand and M. Morrison for reviewing the manuscript, W. Dansgaard and H. Clausen for provision of and help in interpreting the oxygen isotope measurements and E. Saltzman for the methane sulphonic acid data. This research was supported by the Electric Power Research Institute, the US Environmental Protection Agency, le Centre National de la Recherche Scientifique and the NSF. Permission to work in Greenland was granted by the Commission for Scientific Research in Greenland.

Changes in submarine hydrothermal ^3He /heat ratios as an indicator of magmatic/tectonic activity

Edward T. Baker* & John E. Lupton†

* Pacific Marine Environmental Laboratory, National Oceanic and Atmospheric Administration, 7600 Sand Point Way NE, Seattle, Washington 98115, USA

† Marine Science Institute and Department of Geological Sciences, University of California, Santa Barbara, California 93106, USA

AN important question in submarine hydrothermal research concerns the connection between hydrothermal discharge from a spreading centre and variations in local magmatic and tectonic activity. Because it is likely that tectonic stretching and concomitant shallow magmatic activity triggered the cataclysmic venting that created the Juan de Fuca Ridge 'megaplumes'^{1–3}, we have for three years monitored the ^3He concentration and temperature anomaly of the underlying steady-state plume at the site of the original megaplume. We report here that the apparent ^3He /heat ratio in the steady-state plume has progressively decreased from 4.4 to 2.4 to $1.3 \times 10^{-12} \text{ cm}^3 \text{ STP cal}^{-1}$, changing from a uniquely high ratio to one characteristic of established vent fields on other ridge segments^{4–8}. We propose that the initially high ^3He /heat ratio, sampled within days of the megaplume eruption, resulted from magma degassing into a hydrothermal circulation system of high permeability and short fluid residence time. Thus, high ^3He /heat ratios may indicate venting created or profoundly perturbed by a magmatic–tectonic event, and lower ratios may typify systems at equilibrium.

When the megaplume at the north end of the Cleft segment of the Juan de Fuca Ridge was discovered in August 1986, an extensive steady-state hydrothermal plume was also found at the same location^{1,9}. The steady-state plume has been annually

mapped by near-bottom conductivity–temperature–depth transmissometer (CTDT) tows and casts, and sampled for He and other constituents using rosette-mounted Niskin bottles. The temperature anomaly (ΔT) of the plume relative to ambient water of the same potential density is calculated using the formula¹⁰

$$\Delta T = (\theta + m\sigma_\theta) - b$$

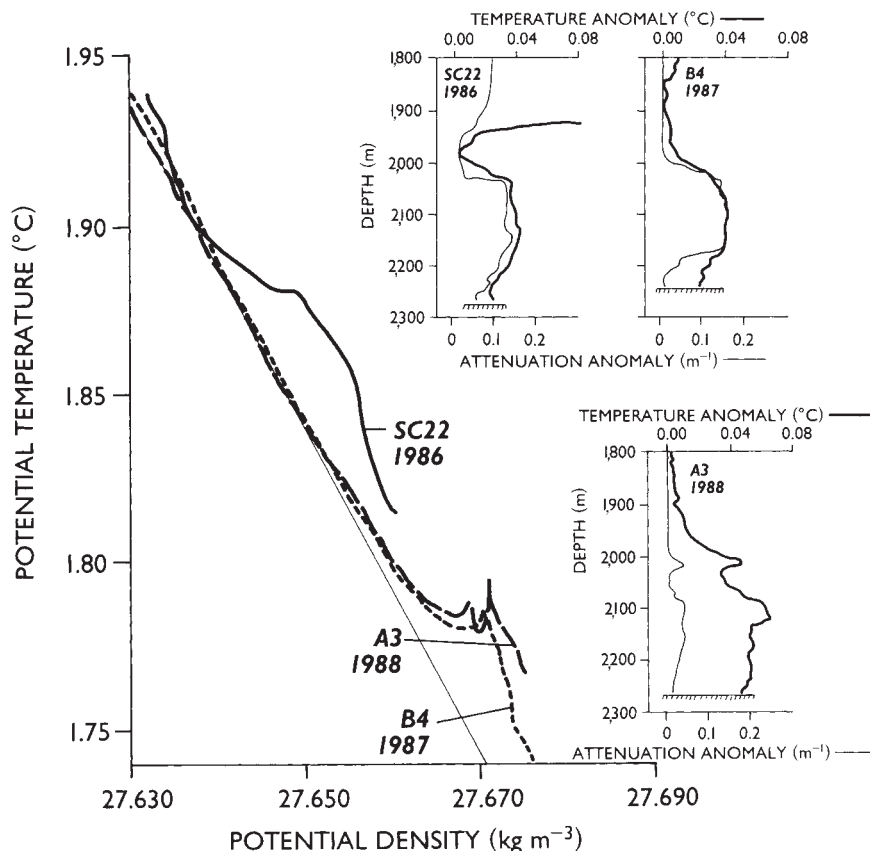
where θ and σ_θ are the potential temperature and potential density, respectively, in the plume, and m and b are the slope and intercept, respectively, of the linear regression of the θ – σ_θ curve of water immediately above the plume where the hydrographic effect of hydrothermal emissions is negligible (Fig. 1). The slope m is constant at $-4.865^\circ\text{C m}^3 \text{ Kg}^{-1}$ each year, whereas the intercept b is adjusted to compensate for changes in σ_θ arising from annual variations of $\pm 0.002\%$ in the salinity calibration. The calculated ΔT values may be an underestimate of the true hydrothermal heating by as much as a factor of two owing to cooling of the plume by entrainment of cold bottom water^{2,11}, and hydrographic aliasing if vent waters have a salinity substantially different from that of ambient sea water¹². Despite some uncertainty in the absolute value of ΔT , we assume that the year-to-year values are correct relative to each other because the consistency of the ambient hydrographic structure and the rise height of the plume (Fig. 1) implies a consistency of plume entrainment processes and vent-fluid salinity.

Figure 2 shows areal maps of ΔT in the steady-state plume created by plotting and contouring maximum ΔT values along the tow track-lines and at the vertical cast locations. The size and intensity of the ΔT plume indicate that venting extends more than 20 km along the ridge axis with a vigour comparable to the vent field at 48°N on the Endeavour segment of the Juan de Fuca Ridge, where the heat flux is calculated to be $1.5 \times 10^9 \text{ W}$ (refs 4, 13). When the megaplume vent field was discovered in 1986, ΔT values $\geq 0.04^\circ\text{C}$ extended from a few kilometres west of the axial valley to the limits of the survey area to the east and north (Fig. 2a). Plume mapping in 1986 was not detailed enough to locate precisely the vent field creating the plume, but the fact that the highest light-attenuation anomalies were found in the axial valley near station SC22 suggests that the source was in the axial valley and the plume distribution was the result of advection to the east.

In 1987 and 1988 the ΔT plumes were less extensive and more symmetrical about the axial valley (Fig. 2b, c). Temperature anomalies in the axial valley averaged 0.03 – 0.04°C with localized highs reaching 0.06°C . Although quantitative flux estimates cannot be derived from plume maps alone, it seems likely that the release of the August 1986 megaplume was accompanied by a higher heat and mass flux in the underlying steady-state plume than has since been characteristic of this vent field.

Unequivocal evidence for temporal variability in hydrothermal processes at this site comes from the progressive decrease in the $^3\text{He}/\Delta T$ ratio of the steady-state plume (Fig. 3). In August 1986, the $^3\text{He}/\Delta T$ ratio in the plume was $(4.4 \pm 0.64) \times 10^{-12} \text{ cm}^3 \text{ STP g}^{-1}^\circ\text{C}^{-1}$ (1σ uncertainty), the highest ratio yet reported for hydrothermal emissions on a mid-ocean ridge and a factor of 14 higher than the $^3\text{He}/\Delta T$ ratio in the overlying megaplume⁹. Concentrations of ^3He in the plume were as high as $0.30 \times 10^{-12} \text{ cm}^3 \text{ STP g}^{-1}$ ($\delta^3\text{He} = 238\%$; $\delta^3\text{He} = [({}^3\text{He}/{}^4\text{He})_{\text{sample}}/({}^3\text{He}/{}^4\text{He})_{\text{air}}] - 1$). In September 1987 the ratio in the steady-state plume decreased to $(2.4 \pm 0.14) \times 10^{-12} \text{ cm}^3 \text{ STP g}^{-1}^\circ\text{C}^{-1}$ (1σ), a factor of 1.8 below the 1986 value. By September 1988 the ratio had decreased further, to $(1.3 \pm 0.1) \times 10^{-12} \text{ cm}^3 \text{ STP g}^{-1}^\circ\text{C}^{-1}$ (1σ), a factor of 3.4 below the 1986 value. The yearly decreases apparently resulted from a decreasing concentration of ^3He in the plume, as the range of ΔT values is about the same each year. In the following discussion we use the conservative assumption that hydrographic cooling and vent-fluid salinity changes had a negligible influence on the observed ΔT values, so that the $^3\text{He}/\Delta T$ ratios in the plumes are equivalent to the

FIG. 1 Potential density plotted against potential temperature for selected axial valley CTD casts in 1986, 1987 and 1988. Each curve has a slope of $-4.865^{\circ}\text{C m}^3 \text{Kg}^{-1}$ (shown by the straight line) except where hydrothermally affected. 1986 casts in the axial valley sampled only a thin horizon of ambient water ($\sim 27.635\text{--}27.640 \sigma_{\theta}$) between the steady-state plume and the overlying megaplume. Density of the water in the axial valley was significantly lower in 1986 because of enhanced vertical mixing of the lowermost 1,000 m by the megaplume discharge. Such vertical mixing does not affect the ambient $\theta\text{--}\sigma_{\theta}$ relationship. The maximum temperature anomaly, defined as the temperature deviation from the background trend along an isopycnal, was about the same on each cast. Insets show vertical profiles of the temperature and light-attenuation (proportional to the concentration of fine-grained hydrothermal precipitates) anomalies for each cast. Increased anomalies above 1,950 m in 1986 indicate the megaplume presence. Depth of the steady-state plume maximum was $\sim 2,100$ m each year. For each cast the light-attenuation and temperature anomalies go to zero at the same depth, supporting our interpretation of the vertical distribution of temperature anomaly.



^3He /heat ratios in the original fluids. We cannot verify this because no high-temperature vents have yet been located at this site.

We propose that the observed ^3He /heat trend represents accelerated degassing from a magma body whose solidification rate was abruptly increased either by vertical intrusion into cooler crust or by a local deepening of the boundary between brittle and ductile deformation. Sub-surface releases of ^3He have been reported at magmatically active terrestrial sites in Japan^{14,15} and Long Valley Caldera in California¹⁶ in association with earthquakes and local faulting. Two degassing processes can enhance the expulsion of He from a magma body and

thereby temporarily increase the ^3He /heat ratio of the surrounding hydrothermal fluids: pressure release or crystal-melt fractionation.

Because of the low and pressure-dependent solubility of CO_2 in silicate liquids¹⁷, CO_2 exsolution forms vesicles in a rising magma. Basalts from the Juan de Fuca Ridge have a mean vesicularity, of $\sim 1\%$ (ref. 18). Determinations of the solubility constant of He in a tholeiitic basalt melt¹⁹ show that for 1% vesicularity, $\geq 60\%$ of the total He is contained in vesicles and is thus more readily available for loss from the melt. The discovery of basalts with $[\text{He}] > 50 \times 10^{-6} \text{ cm}^3 \text{ STP g}^{-1}$, ten times larger than that of typical mid-ocean ridge basalts²⁰, suggests

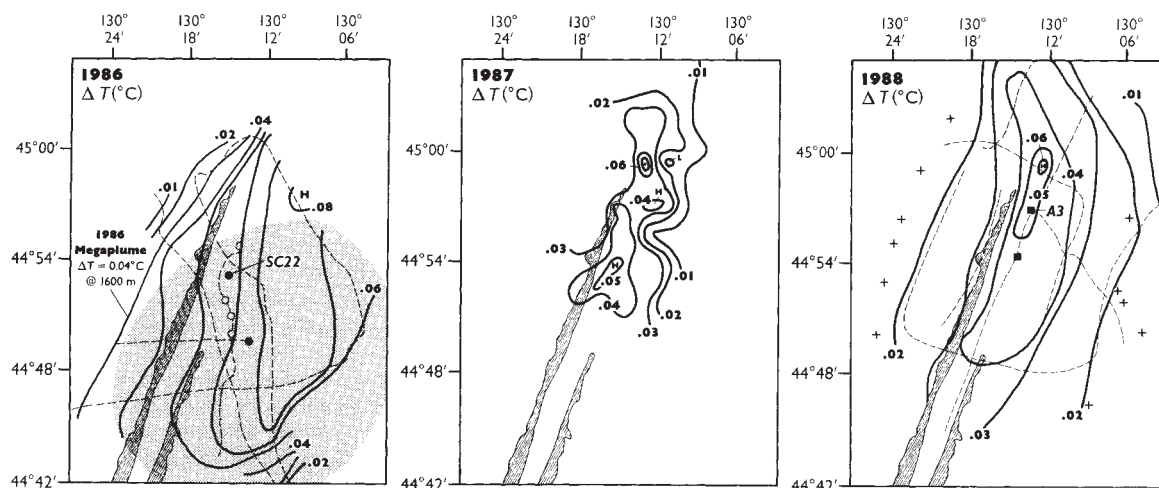


FIG. 2 Areal maps of the steady-state plume as described by the maximum temperature anomaly between 2,000 m and 2,200 m along CTD tow tracks (dashed lines) and vertical casts (crosses or solid symbols) in 1986, 1987 and 1988. ^3He samples were collected during tows (open symbols) or on vertical casts (solid symbols); the locations of the Fig. 1 profiles are indicated.

Hatched areas are the walls of the axial valley as defined by the 2,200-m bathymetric contour. In each year the plume was ~ 200 m thick and centred ~ 150 m above the floor of the axial valley (see Fig. 1). The plume was more extensive in 1986 than in either 1987 or 1988. The size and location of the 1986 megaplume is shown by shading.

that a large proportion of magmatic He is commonly lost by pre-eruptive degassing.

As the intrusion solidifies, He is progressively concentrated in the remaining melt by crystal-melt fractionation because the distribution coefficient for He between crystals and a basaltic melt is ≤ 0.1 – 0.01 (refs 21–23). Freezing of a basaltic melt at a liquidus temperature of $1,150^\circ\text{C}$ excludes 90–99% of the He from individual crystals but liberates only $\sim 100\text{ cal g}^{-1}$, $\sim 25\%$ of the total heat lost on cooling to seawater temperature. A thick and slowly cooling intrusion might thus supply an elevated, but decreasing, $^3\text{He}/\text{heat}$ flux for several years.

An alternative to magma intrusion is the incremental deepening of the cracking front²⁴ above a magma chamber (J. R. Delaney, personal communication). A sudden increase in the permeability of the crust, perhaps caused by tectonic stretching, might allow deeper penetration of cold sea water and an accelerating solidification of the magma.

Lupton *et al.*⁹ have suggested that $^3\text{He}/\text{heat}$ variations in mid-ocean-ridge vent systems do not arise from geographic inhomogeneities in parent magmas but are symptomatic of dynamic conditions such as the age and permeability of the circulation system feeding the vent field. The changing $^3\text{He}/\text{heat}$ ratios reported here support that prediction and may thus describe a changing hydrothermal circulation system. A sudden permeability increase caused by crustal fracturing associated with magma solidification could accelerate the release of pre-equilibrated fluids having a 'normal' $^3\text{He}/\text{heat}$ ratio, causing a megaplume event. Fluids remaining in the now rejuvenated, highly permeable, circulation system are enriched with degassing He faster than they are heated, causing $^3\text{He}/\text{heat}$ ratios to exceed the theoretical ratio of $\sim 2 \times 10^{-12}\text{ cm}^3\text{ STP cal}^{-1}$ expected for upper mantle magma⁹. Indeed, the $^3\text{He}/\text{heat}$ ratio in the steady-state plume immediately after the megaplume disruption was more twice this theoretical ratio.

As the system ages, circulation approaches equilibrium and the fluid pathways become more tortuous as a result of mineral precipitation. $^3\text{He}/\text{heat}$ ratios decline as the residence time of fluids in the circulation system increases and the extraction rates for heat and He converge. The compositionally unchanging fluids at the 21°N vent field on the East Pacific Rise²⁵ have a

low $^3\text{He}/\text{heat}$ ratio ($0.4 \times 10^{-12}\text{ cm}^3\text{ STP cal}^{-1}$) (refs 6, 8) that may reflect equilibrium conditions. Recent studies of Icelandic geothermal systems²⁶ and the degassing behaviour of mid-ocean-ridge magma²⁰ also suggest that $^3\text{He}/\text{heat}$ ratios can decline as a magmatic system ages. Based on our observations, the time-scale for a return to equilibrium conditions can be of the order of a few years, in agreement with radiochemical estimates of the residence time of hydrothermal fluids in the crust²⁷. $\square$

Received 5 April; accepted 15 June 1990.

1. Baker, E. T., Massoth, G. J. & Feely, R. A. *Nature* **329**, 149–151 (1987).
2. Baker, E. T. *et al.* *J. geophys. Res.* **94**, 9237–9250 (1989).
3. Cann, J. R. & Strens, M. R. *J. geophys. Res.* **94**, 12227–12237 (1989).
4. Rosenberg, N. D. *et al.* *Nature* **334**, 604–607 (1988).
5. Jenkins, W. J., Edmond, J. M. & Corliss, J. B. *Nature* **272**, 156–158 (1978).
6. Welhan, J. A. & Craig, H. in *Hydrothermal Processes at Seafloor Spreading Centers* (eds Rona, P. A., Bostrom, K., Laubier, L. & Smith, K. L. Jr) 391–409 (Plenum, New York, 1983).
7. Merlivat, L., Pineau, F. & Javoy, M. *Earth planet. Sci. Lett.* **84**, 100–108 (1987).
8. Lupton, J. E. *et al.* *Earth planet. Sci. Lett.* **50**, 115–127 (1980).
9. Lupton, J. E., Baker, E. T. & Massoth, G. J. *Nature* **337**, 161–164 (1989).
10. Lupton, J. E., Delaney, J. R., Johnson, H. P. & Tivey, M. K. *Nature* **316**, 621–623 (1985).
11. Speer, K. G. & Rona, P. A. *J. geophys. Res.* **94**, 6213–6220 (1989).
12. McDuff, R. E. *Eos* **69**, 1497 (abstr.) (1988).
13. Baker, E. T. & Massoth, G. J. *Earth planet. Sci. Lett.* **85**, 59–73 (1987).
14. Wakita, H. *et al.* *Science* **200**, 430–432 (1978).
15. Sano, Y., Nakamura, Y., Wakita, H., Notsu, K. & Kobayashi, Y. *J. geophys. Res.* **91**, 12291–12295 (1986).
16. Welhan, J. A., Poreda, R. J., Rison, W. & Craig, H. *J. Volcanol. geotherm. Res.* **34**, 201–209 (1988).
17. Stolper, E. & Holloway, J. R. *Earth planet. Sci. Lett.* **87**, 397–408 (1988).
18. Dixon, J. E., Stolper, E. & Delaney, J. R. *Earth planet. Sci. Lett.* **90**, 87–104 (1988).
19. Jambon, A., Weber, H. & Braun, O. *Geochim. cosmochim. Acta* **50**, 401–408 (1986).
20. Sarda, P. & Graham, D. *Earth planet. Sci. Lett.* **97**, 268–289 (1990).
21. Kirsten, T. *J. geophys. Res.* **73**, 2807–2810 (1968).
22. Kurz, M. D., Jenkins, W. J., Schilling, J. G. & Hart, S. R. *Earth planet. Sci. Lett.* **58**, 1–14 (1982).
23. Hyagon, H. & Ozima, M. *Geochim. cosmochim. Acta* **50**, 2045–2057 (1986).
24. Lister, C. R. B. in *Hydrothermal processes at Seafloor Spreading Centers* (eds Rona, P. A., Bostrom, K., Laubier, L. & Smith, K. L. Jr) 141–168 (Plenum, New York, 1983).
25. Campbell, A. C. *et al.* *J. geophys. Res.* **93**, 4537–4550 (1988).
26. Poreda, R. J., Arnorsson, S. & Craig, H. *Geochim. cosmochim. Acta* (in the press).
27. Kadko, D. & Moore, W. *Geochim. cosmochim. Acta* **52**, 659–668 (1988).

ACKNOWLEDGEMENTS. We thank G. Massoth for collecting the He samples, S. Walker for processing the CTD data, K. Thornberry, D. Dion and A. Faizullah for the He isotope measurements, and D. Graham for comments on the manuscript. This work was supported by the NOAA and the NSF.

Microorganisms associated with chromosome destruction and reproductive isolation between two insect species

Johannes A. J. Breeuwer & John H. Werren

Department of Biology, University of Rochester, Rochester, New York 14627, USA

MICROORGANISMS have been implicated in causing cytoplasmic incompatibility in a variety of insect species, including mosquitoes, fruitflies, beetles and wasps^{1–17}. The effect is typically unidirectional: incompatible crosses produce no progeny^{1–11} or sterile males^{12–14}, whereas the reciprocal crosses produce normal progeny. The parasitic wasp *Nasonia vitripennis* is one of the few species in which the cytogenetic mechanism of incompatibility is known. In this species the paternal chromosome set forms a tangled mass in a fertilized egg and is eventually lost¹⁶. Here we report that cytoplasmic microorganisms are associated with complete bidirectional incompatibility between *N. vitripennis* and a closely related sympatric species, *N. giraulti*. Microorganisms can be seen in the eggs of both species. Hybrid offspring are normally not produced in crosses between the two species, but do occur after elimination of the microorganisms by antibiotic treatment. A cytogenetic and genetic study shows that bidirectional interspecific incompatibility is due to improper condensation of the paternal chromosomes. Microorganism-mediated reproductive isolation is of interest because it could provide a rapid mode of speciation^{18,19}. The

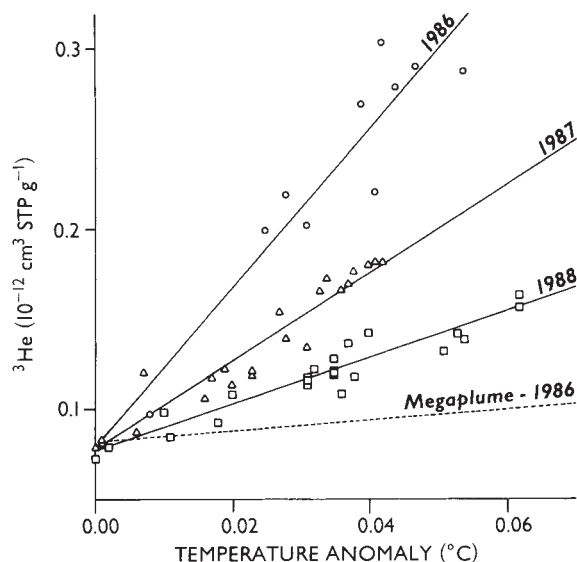


FIG. 3 ^3He concentration plotted against temperature anomaly for samples in steady-state plume from 1986, 1987 and 1988. Sample locations shown in Fig. 2. Least-squares trend of 1986 megaplume samples (which had a maximum temperature anomaly of $\sim 0.26^\circ\text{C}$) shown for comparison. Least-squares regression shows that the $^3\text{He}/\Delta T$ ratio decreased from 4.4 to 2.4 to $1.3 \times 10^{-12}\text{ cm}^3\text{ STP g}^{-1}\text{ }^\circ\text{C}^{-1}$ from 1986 to 1988. A *t*-test analysis indicates that these ratios are all significantly different from each other at the 95% confidence level.

TABLE 1 Per cent hybrids (females)* and offspring number† for crosses using Symbiont and Asymbiont strains

Male parent	Female parent			
	Symbiont		Asymbiont	
	Nv	Ng	Nv	Ng
Symbiont				
Nv	66 ± 11%	0 ± 0%	0 ± 0%	0 ± 0%
Ng	0 ± 0%	84 ± 4%	0 ± 0%	0 ± 0%
Asymbiont				
Nv	69 ± 8%	76 ± 8%	75 ± 4%	80 ± 9%
Ng	20 ± 22%	82 ± 25%	19 ± 24%	83 ± 7%
Symbiont				
Nv	45.9 ± 8.3 (27)	34.2 ± 8.0 (30)	54.8 ± 8.8 (10)	33.6 ± 7.7 (12)
Ng	47.9 ± 7.8 (28)	34.9 ± 8.6 (24)	41.6 ± 11.4 (24)	25.8 ± 10.2 (17)
Asymbiont				
Nv	39.4 ± 7.2 (5)	25.2 ± 8.1 (28)	40.0 ± 10.5 (11)	29.1 ± 6.5 (10)
Ng	42.5 ± 8.3 (15)	39.1 ± 7.2 (12)	39.9 ± 10.6 (20)	37.1 ± 9.2 (13)

* The per cent of hybrids (females) ($\pm$ s.d.) are given in the upper part of the table for crosses using Symbiont (S) and Asymbiont (A) strains.

† Offspring number ($\pm$ s.d.) are shown in the lower part of the table for crosses using Symbiont and Asymbiont strains.

Sample sizes (shown in parentheses in the lower part of the table) are number of replicates in which four females are given four hosts. Recall that in haplodiploid organisms, only females are genetic hybrids, whereas the male is uniparentally derived. Offspring numbers are not reduced in interspecific crosses: NgS δ $\times$ NvS δ versus NvS δ $\times$ NvS δ ($P > 0.60$) and NvS δ $\times$ NgS δ versus NgS δ $\times$ NgS δ ($P > 0.60$; Mann-Whitney U -test). Hybrids are not produced in S δ $\times$ S δ crosses, but are produced in A δ $\times$ S δ crosses and A δ $\times$ A δ crosses. A proportion of hybrids are produced in NgA δ $\times$ NvA and NvS δ crosses that is significantly lower than in reciprocal crosses ($P < 0.001$; Mann-Whitney U -test). This may be due to a lower mating propensity of Nv δ for Ng δ , rather than to differences in the degree of compatibility.

An Asymbiont strain of Ng was produced by feeding (NgVa002) adult females a solution of 1 mg ml⁻¹ tetracycline in 10% sucrose and then allowing the females to lay eggs for 48 h. The procedure was repeated with the progeny for three more generations, at which time no bacteria could be detected within the eggs of this Ng Asymbiont line. The experiments were conducted 2 months (4 generations) after curing the Ng strain. An Nv asymbiont strain was used that had been cured of the Symbiont two years previously. Examination of eggs showed that the symbionts were still absent in both strains. Virgins were allowed to mate for 24 h (2 males $\times$ 4 females). After mating, females were placed on four hosts (*Sarcophaga bullata* pupa) and allowed to lay eggs. The subsequent number of offspring and sex ratio were scored.

mechanism of incompatibility in *Nasonia* is also of interest as a potential tool for studying chromosome imprinting and chromosome condensation.

Nasonia vitripennis is a small wasp which lays its eggs in the pupae of various fly species^{20,21}. As in other bees and wasps, *N. vitripennis* has haplodiploid sex determination. Males typically develop from unfertilized (haploid) eggs, whereas females develop from fertilized (diploid) eggs. Three species of *Nasonia* have been described that can be distinguished on morphological, behavioural and genetic criteria²². *N. longicornis* occurs in western North America, *N. giraulti* occurs in eastern North America and *N. vitripennis* is cosmopolitan. *N. vitripennis* is found micro-sympatrically with each of its sibling species. Indeed, different species have been observed to emerge from the same patch of host pupae and even from the same host puparia²². Therefore, the potential for interspecific hybridization is real.

A study was originally undertaken to determine whether crosses between *N. vitripennis* (Nv) and *N. giraulti* (Ng) produced fertile hybrid offspring. Strains of each species differ in the degree of interspecific premating isolation (J.H.W. and M. Riley, unpublished results). Therefore we used an Ng strain (NgVa002) for initial studies of hybridization between Nv and Ng that readily mates with Nv, and a standard Nv strain (Lab II). Although matings between the two species readily occurred, interspecific matings yielded only all-male progeny. Because only females are hybrids in haplodiploid species, no hybrid offspring were produced.

All-male progeny could have arisen from these crosses for at least five reasons: (1) absence of copulation (virgin females produce all-male offspring); (2) inadequate insemination or failure of the sperm to reach the spermatheca; (3) failure of sperm to fertilize eggs; (4) post-fertilization incompatibility, resulting in loss of paternal chromosomes, or (5) hybrid (female) mortality. Possibilities (1) and (2) are eliminated because copulations were observed in interspecific matings, and dissection of females after mating revealed the presence of sperm in the spermatheca. Possibility (5) is eliminated because offspring numbers were not significantly reduced in interspecific matings (Table 1).

Saul and colleagues¹⁵⁻¹⁷ previously discovered unidirectional cytoplasmic incompatibility between laboratory strains of *N. vitripennis*, and demonstrated cytogenetically that the paternal chromosomes fail to condense properly and are eventually lost in incompatible crosses. Because of haplodiploid sex determination, the incompatible crosses produce all-male progeny. They further established that incompatibility was caused by an agent that could be eliminated by tetracycline treatments and reinoculated into previously cured lines¹⁵.

We therefore undertook a series of experiments to determine whether the absence of hybrid progeny in crosses between these species is caused by cytoplasmic incompatibility. First, we conducted a cytogenetic examination to follow the developmental events after egg fertilization in interspecific crosses (for procedures, see Fig. 1 legend and ref. 23). In both intra- and interspecific fertilized eggs, spermatozoa were easily detected. But by the first mitotic (cleavage) division, a dramatic difference was found between fertilized eggs from intraspecific and interspecific crosses. A normal diploid complement was typically present in eggs from intraspecific matings, but in fertilized eggs from interspecific matings, one set of chromosomes formed a diffuse tangled chromatin mass (Fig. 1). Use of an eye-colour mutant confirmed that the paternal chromosomes were destroyed in interspecific crosses. Bacteria can be seen in the eggs of normal (Symbiont) strains of both species, but are absent in antibiotic-cured (Asymbiont) strains (see Fig. 1 legend for method). The coccoid bacteria are clustered at the posterior pole of the egg (Fig. 1). This is the region that typically develops into germ-line tissues in insects.

To determine whether the symbiont microorganisms have a role in reproductive isolation between the sibling species, intra- and interspecific crosses were performed using Symbiont and Asymbiont strains. For each species, the Symbiont strain used in the experiments is the same one from which the Asymbiont strain was derived, so they are genetically identical. Results (Table 1) show that within each species, Symbiont males are incompatible with Asymbiont females, whereas the reciprocal cross is compatible. This is the same unidirectional cytoplasmic incompatibility pattern found within many other insect species^{1-11,15-17}.

Interspecific crosses yield a dramatic result. Whereas crosses between the Symbiont (standard) strains of the two species result in all-male offspring, crosses using Asymbiont males of one species with either Symbiont or Asymbiont females from the other species results in production of hybrid (female) offspring. Thus, we conclude that elimination of the microorganism allows the two species to produce viable hybrids. Hybrid (female)

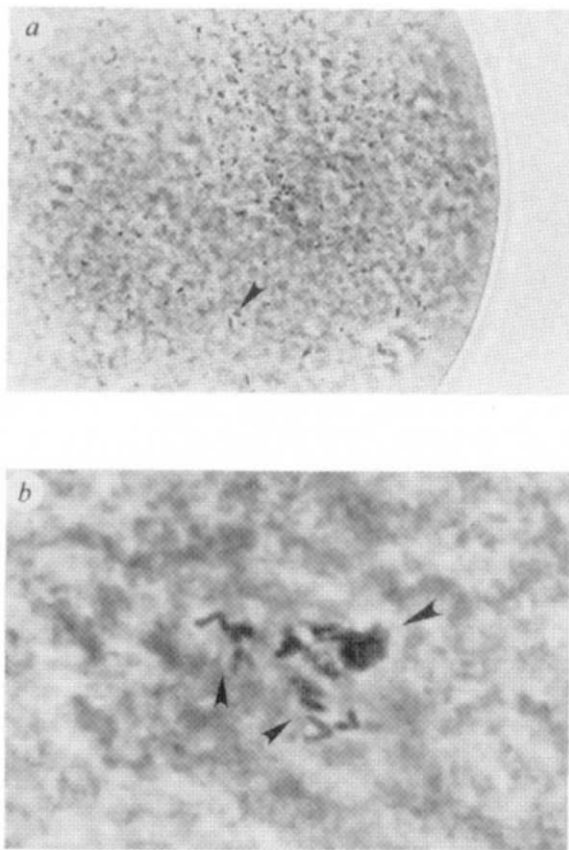


FIG. 1 *a*, Cytoplasmic microorganisms within an egg of *N. giraulti*. Bacteria are present throughout the posterior pole of the egg. Arrow indicates a dividing bacterium. These bacteria are absent in tetracyclin-treated strains. Although the species of microorganism is unknown, cytoplasmic incompatibility in several other insects is caused by rickettsia⁵⁻¹¹. By counting bacteria in thirteen 0.01 mm $\times$ 0.01 mm columns through the egg and extrapolating to the total region containing bacteria within the egg, numbers of bacteria per standard Nv (Lab II) eggs are crudely estimated to be $1,903 \pm 476$ s.d. ($n=6$). For a standard Ng (Va002), strain counts are $2,143 \pm 1,017$ s.d. ($n=15$). *b*, The appearance of improperly condensed chromosomes in anaphase of the first mitotic division of an incompatible cross between *N. giraulti* female and *N. vitripennis* male. The large arrow indicates the improperly condensed chromosome set. Two smaller arrows indicate dividing chromosomes. Based upon the genotype of male offspring resulting from such crosses, it can be concluded that it is the paternal chromosome set that fails to condense properly and is eventually lost. These cytogenetic events are similar to those described for unidirectionally incompatible crosses between *N. vitripennis* strains¹⁵⁻¹⁷.

METHODS. *a*, Eggs, collected between 0 and 70 min after oviposition by the wasp, were placed in Carnoy's fixative (acetic acid:chloroform:95% ethanol in proportions 1:2.5:3). One to three days after fixation, eggs were transferred to a drop of 70% ethanol and mechanically stripped of their chorion and then stained in 2% lacmoid. *b*, Conditions as in *a*, except 2% aceto-lactic orcein was used for staining.

offspring from both interspecific crosses are fertile, although there is increased mortality among their offspring (data not shown). Therefore, the species have diverged sufficiently genetically to produce partial hybrid breakdown.

It has been suggested that Symbionts that cause cytoplasmic incompatibility play a part in the process of speciation by preventing gene flow between populations^{18,19}. Caspari and Watson²⁴, however, showed with a population model that unidirectional incompatibility cannot easily result in reproductive isolation because of the rapid spread of the symbiont cytotype. By contrast, symbionts causing bidirectional incompatibility can lead to reproductive isolation between populations and ultimately to speciation.

The symbiont microorganisms may have facilitated speciation in *Nasonia* by causing reproductive isolation. Whether they still actively maintain reproductive isolation between these species depends on the frequency of interspecific matings. The two species are now sympatric over much of their range. Strains of the two species differ in degree of premating isolation (under laboratory conditions), suggesting that this character is actively evolving, perhaps in response to incompatibility. But we do not know whether interspecific mating is frequent in nature.

The bidirectional incompatibility between *Nasonia* species described here causes complete reproductive isolation that can be removed by eliminating an associated microorganism. Other examples of bidirectional incompatibility occur between strains of *Culex pipiens*^{25,26}, and between semi-species in *Drosophila paulistorum*¹²⁻¹⁴. The number of documented cases of cytoplasmic incompatibility within insect species is rapidly growing as new species are examined¹⁻¹⁷. On the basis of our findings, we suggest that cytoplasmic incompatibility between closely related arthropod species may be more widespread and significant than has previously been believed²⁴.

The biochemical mechanisms by which a symbiont can cause improper condensation of its eukaryotic host's chromosomes is

an unknown and intriguing aspect of this system. Chromosomal imprinting is likely to be involved. *N. vitripennis* harbours (in some populations) a supernumerary chromosome which also causes destruction of the paternal chromosomes^{23,27}. In that case the chromosomes appear to hypercondense, whereas cytoplasmic incompatibility results in undercondensation. These non-mendelian elements could prove useful in studying molecular mechanisms of chromosome imprinting and chromosome condensation, as well as in elucidating modes of speciation. □

Received 4 December 1989; accepted 25 May 1990.

- Hoffman, A. A., Turelli, M. & Simmons, G. M. *Evolution* **40**, 692-701 (1986).
- Hoffman, A. A. *Entomol. Exp. Appl.* **48**, 61-67 (1988).
- Hsiao, T. H. & Hsiao, C. *Entomol. Exp. Appl.* **37**, 155-159 (1985).
- Wade, M. J. & Stevens, L. *Science* **227**, 527-528 (1989).
- Binnington, K. C. & Hoffmann, A. A. *J. Invert. Pathol.* **54**, 344-352 (1989).
- Trpis, M., Perrone, J. B., Reissig, M. & Parker, K. L. *J. Hered.* **72**, 313-317 (1981).
- Yen, J. H. & Barr, A. R. *Nature* **232**, 657-658 (1971).
- Hsiao, T. H. & Hsiao, C. *J. Invert. Pathol.* **45**, 244-246 (1985).
- O'Neill, S. L. *J. Invert. Pathol.* **53**, 132-134 (1989).
- Yen, J. H. & Barr, A. R. *J. Invert. Pathol.* **22**, 242-250 (1973).
- Kellen, W. R., Hoffman, D. F. & Kwock, R. A. *J. Invert. Pathol.* **37**, 273-283 (1981).
- Dobzhansky, T. & Paulovsky, O. *Genetics* **55**, 141-156 (1967).
- Ehrman, L. & Kernaghan, R. P. *J. Hered.* **62**, 67-71 (1971).
- Williamson, D. L., Ehrman, L. & Kernaghan, R. P. *Proc. natn. Acad. Sci. U.S.A.* **68**, 2158-2160 (1971).
- Richardson, P. M., Holmes, W. P. & Saul II, G. B. *J. Invert. Pathol.* **50**, 176-183 (1987).
- Ryan, S. L. & Saul II, G. B. *Molec. gen. Genet.* **103**, 29-36 (1986).
- Conner, G. W. & Saul II, G. B. *J. Hered.* **77**, 211-213 (1986).
- Laven, H., Wright, J. W. & Pal, R. in *Genetics of Insect Vectors of Diseases* (Elsevier, Amsterdam, 1967).
- Thompson, J. N. *Biol. J. Linn. Soc.* **32**, 385-393 (1987).
- Whiting, A. R. *Q. Rev. Biol.* **42**, 333-406 (1967).
- Werren, J. H. *Evolution* **37**, 116-124 (1983).
- Darling, D. C. & Werren, J. H. *Ann. Entomol. Soc. Amer.* **83**, 352-370 (1990).
- Werren, J. H., Nur, U. & Eickbush, D. *Nature* **327**, 75-76 (1987).
- Caspari, E. & Watson, G. S. *Evolution* **13**, 568-570 (1959).
- Laven, H. Z. *Vererbungslehre* **88**, 478-516 (1987).
- Subbarao, S. K., Krishnamurthy, B. S., Curtis, C. F., Adak, T. & Chandras, R. K. *Genetics* **87**, 381-390 (1977).
- Nur, U., Werren, J. H., Eickbush, D. G., Burke, W. B. & Eickbush, T. H. *Science* **240**, 512-514 (1988).

ACKNOWLEDGEMENTS. We thank L. Beukeboom, D. Eickbush, D. Ferris, J. Jaenike, D. Miller, U. Nur, M. Riley, R. Stouthamer and M. Vig for comments and assistance. Support was provided by the NSF, the NIH and Donders Fonds.

Structure of a cannabinoid receptor and functional expression of the cloned cDNA

Lisa A. Matsuda, Stephen J. Lolait,
Michael J. Brownstein, Alice C. Young
& Tom I. Bonner

Laboratory of Cell Biology, National Institutes of Mental Health, Bethesda, Maryland 20892, USA

MARIJUANA and many of its constituent cannabinoids influence the central nervous system (CNS) in a complex and dose-dependent manner^{1,2}. Although CNS depression and analgesia are well documented effects of the cannabinoids, the mechanisms responsible for these and other cannabinoid-induced effects are not so far known³. The hydrophobic nature of these substances has suggested that cannabinoids resemble anaesthetic agents in their action, that is, they nonspecifically disrupt cellular membranes. Recent evidence, however, has supported a mechanism involving a G protein-coupled receptor found in brain and neural cell lines, and which inhibits adenylate cyclase activity in a dose-dependent, stereoselective and pertussis toxin-sensitive manner⁴⁻⁷. Also, the receptor is more responsive to psychoactive cannabinoids than to non-psychoactive cannabinoids⁸. Here we report the cloning and expression of a complementary DNA that encodes a G protein-coupled receptor with all of these properties. Its messenger RNA is found in cell lines and regions of the brain that have cannabinoid receptors. These findings suggest that this protein is involved in cannabinoid-induced CNS effects (including alterations in mood and cognition) experienced by users of marijuana.

In our attempts to clone novel receptors, we isolated a cDNA (SKR6) from a rat cerebral cortex cDNA library, using an oligonucleotide probe derived from the sequence of bovine substance-K receptor⁹. The translated sequence of this cDNA identified its 473-amino-acid protein product as a member of the G protein-coupled family of receptors (Fig. 1). Seven hydrophobic domains, numerous residues that are highly conserved among G protein-coupled receptors and several potential glycosylation sites were apparent (Fig. 1). If glycosylated, the relative molecular mass of this receptor would therefore exceed that of 52,823 predicted from its amino-acid constituents. Despite its general similarity to other receptors in this family, the resemblance of SKR6 to the amino-acid sequence of any other receptor was not close enough to allow us to predict either the identity of the receptor's ligand or the coupling system responsible for its signal transduction processes in the cell. Before the identification of SKR6 as a cannabinoid receptor, therefore, many candidate ligands were examined.

Identification of the ligand for SKR6 initially involved screening either SKR6-transfected mammalian cells or *Xenopus* oocytes injected with RNA transcribed from the cDNA *in vitro*. Ligands for receptors that exist on cell lines in which SKR6 mRNA was also found (N18TG-2 or NG108-15 cells; Fig. 2a) were considered strong candidates^{5,10}. In addition, many substances were examined because their receptors and the distribution of SKR6 mRNA (L.A.M., T.I.B. and S.J.L., manuscript in preparation) displayed similar localization patterns in brain. In transfected cells, however, many substances failed to interact with the receptor in radiolabelled ligand binding assays (that is, bradykinin, angiotensin II, neurotensin, cholecystokinin, vasoactive intestinal peptide, adenosine analogues), as well as in assays designed to detect alterations in cyclic AMP production (that is, D-Ala-D-Leu enkephalin, somatostatin, secretin and others at 1 or 10 μ M). In addition, electrophysiological effects in oocytes due to receptor-mediated changes (including those due to increased phosphatidylinositol turnover) were not detected when tested with angiotensin II,

bradykinin, substance P, neuropeptide Y, neurotensin, vasopressin and other ligands at 1 or 10 μ M. Although this strategy for selecting candidate ligands is beset with limitations, the critical findings, which prompted us to examine cannabinoids as ligands for SKR6, included the presence of both cannabinoid receptors^{5,11} and SKR6 mRNA in the same cell lines (Fig. 2a) and the localization of both the receptor^{12,13} and SKR6 mRNA in similar brain areas (Fig. 2b; data not shown).

In Chinese hamster ovary K1 cells stably transfected with SKR6, expression of a cannabinoid-responsive, G protein-coupled receptor was obtained. The major psychoactive cannabinoid found in marijuana (Δ^9 -tetrahydrocannabinol, Δ^9 -THC) and a synthetic analogue with potent analgesic properties (CP 55940) inhibited forskolin-stimulated accumulation of cAMP in a dose-dependent manner (Fig. 3a). In addition, the dose-response curves for the opposite (+) enantiomeric forms of these two cannabinoids indicated this effect was stereoselective. The effector concentration for half-maximum response (EC_{50}) of CP 55940 compared with that of its (+) enantiomer (CP 56667) revealed a >100-fold difference in potencies between these compounds. By contrast, the difference in EC_{50} observed between (+) and (-) Δ^9 -THC was only 50-fold. These data are in general agreement with data for N18TG-2 cell membranes, that is, that the degree of stereoselectivity between various cannabinoid analogues is greater with more potent compounds (such as CP 55940 compared with CP 56667) (ref. 14). As observed in neuroblastomas^{8,14}, none of the cannabinoids inhibited cAMP accumulation by 100 per cent but CP 55940 inhibited the accumulation of cAMP more than Δ^9 -THC. In addition, (-) Δ^8 -THC was less potent than (-) Δ^9 -THC yet affected cAMP to a similar extent (inhibition of 36 versus 39 per cent). Finally, in transfected cells, cannabinol produced only a slight effect on cAMP accumulation, whereas the non-psychoactive cannabinoid, cannabidiol, did not markedly alter cAMP (Fig. 3b).

In N18TG-2 neuroblastomas, the relative potencies of various cannabinoids that inhibit adenylate cyclase correlate well with those of the psychoactive cannabinoids in producing a 'high' in humans⁸. The rank order of potencies for several cannabinoid compounds in SKR6-transfected cells (Fig. 3b) was also similar to that for both the effects in N18TG-2 cell membranes and psychoactive effects in humans^{8,15}: 11-OH Δ^9 -THC > (-) Δ^9 -THC > (-) Δ^8 -THC > cannabinol > cannabidiol. In addition, nabilone, a synthetic cannabinoid analogue marketed for its anti-emetic effects also inhibited cAMP accumulation in SKR6-transfected cells. These cannabinoid-induced responses were probably mediated by the G protein, G_i (ref. 16), as the inhibition of cAMP accumulation was prevented by pretreatment with pertussis toxin (data not shown).

Clearly the dose-dependent, stereoselective and ligand-specific responses of SKR6-transfected cells were those that would be expected from a cannabinoid receptor. These data, along with the work of others, provide evidence for a receptor-mediated mechanism in the effects observed with cannabinoids. Nonetheless, given the substantial amount of research that has focused on the nonspecific actions of these compounds on cellular membranes^{17,18}, one might argue that cannabinoids could considerably compromise the ability of membrane-located receptors to respond correctly to their appropriate ligands. Cannabinoid-induced inhibition of adenylate cyclase activity might then seem to be receptor-mediated but would not be receptor-specific. The lack, however, of cannabinoid-induced inhibition of cAMP accumulation in nontransfected cells (data not shown) demonstrates that these compounds (Δ^9 -THC, 11-OH Δ^9 -THC, nabilone and CP 55940) failed to interact with the endogenous receptors present on CHO cells. Furthermore, when transfected into this same host (CHO cells), neither an α -adrenergic (M. Voigt and C. Felder, personal communication) nor muscarinic receptor⁹ responded to (-) Δ^9 -THC or CP 55940 (Table 1). Both these receptors, however, reduced cAMP

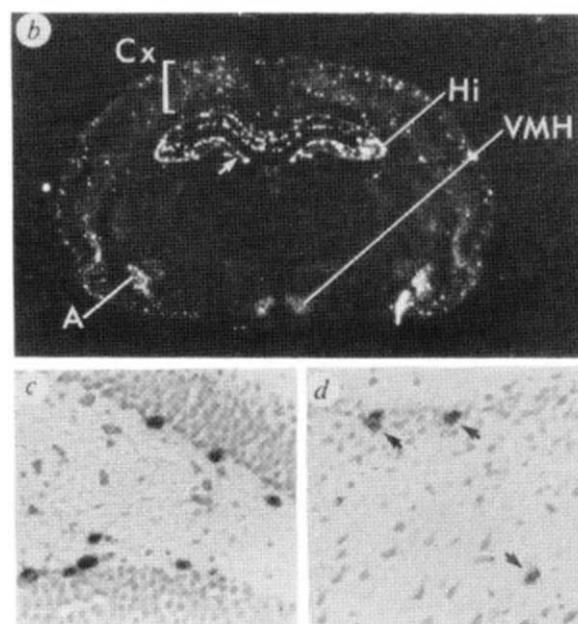
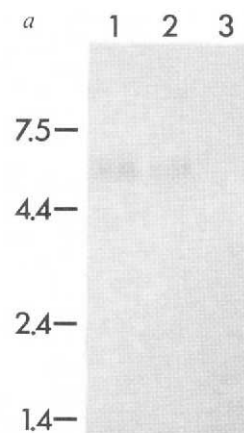
TABLE 1 Cyclic AMP accumulation

Cell line	Receptor/cDNA	Forskolin	Δ^9 THC (100 nM)	CP 55940 (10 nM)	Carbachol/clonidine
CHO	SKR6	100 $\pm$ 4 (12.6 $\pm$ 0.5)	61 $\pm$ 5	—	—
CHO	SKR6	100 $\pm$ 5 (12.1 $\pm$ 1.4)	—	44 $\pm$ 11	—
CHO	muscarinic m2	100 $\pm$ 5 (18.0 $\pm$ 0.9)	104 $\pm$ 8	104 $\pm$ 10	8 $\pm$ 1
CHO	adrenergic α 2d	100 $\pm$ 4 (13.9 $\pm$ 0.5)	100 $\pm$ 4	96 $\pm$ 7	73 $\pm$ 5
N18TG-2	—	100 $\pm$ 10 (44.4 $\pm$ 4.3)	61 $\pm$ 8	16 $\pm$ 2	—
NG108-15	—	100 $\pm$ 4 (320.5 $\pm$ 11.7)	91 $\pm$ 7	57 $\pm$ 3	—

Effect of Δ^9 -THC and CP 55940 on forskolin-stimulated accumulation of cAMP in CHO-K1 cells transfected with SKR6, muscarinic and α -adrenergic receptor cDNAs. Values represent the average accumulation of cAMP $\pm$ s.e.m. as per cent of forskolin-stimulated controls. In each cell line, the effects of the various agonists were examined in three to five experiments (each performed in triplicate). Numbers in parentheses are the absolute values of cAMP as determined by radioimmunoassay (pmol cAMP per 10^6 cells per 5 min). Final concentrations of forskolin were 500 nM for all cell lines except NG108-15; forskolin concentration for this cell line was 250 nM. The muscarinic and adrenergic receptor-transfected cells were assayed under conditions identical to those routinely used to test the SKR6-transfected cells (see Fig. 3). Final concentrations of carbachol (agonist for muscarinic receptors) and clonidine (agonist for α -adrenergic receptors) were 100 μ M and 10 μ M, respectively. Clearly, the extent to which a receptor can inhibit cAMP accumulation varies considerably across different cell lines. The moderate effect of clonidine to inhibit cAMP accumulation reported here is lower than normally observed in this transfected cell line (inhibits cAMP accumulation to 50–25% of forskolin-stimulated control). This difference is due to the bovine serum albumin included in our assay.

FIG. 2 Presence of SKR6 mRNA in cell lines and its localization in rat brain. *a*, Northern analysis of total RNA from N18TG-2 (lane 1), NG108-15 (lane 2) and C6BU-1 (lane 3) cell lines. N18TG-2 and C6BU-1 cells are the neuroblastoma and glioma parents of the NG108-15 hybrid cell line, respectively. The single hybridizing bands present in lanes 1 and 2 are $\sim$ 6 kb. Size markers (kb), on the left. Northern analysis was also performed on both total (10 μ g) and poly(A)⁺ RNA (5 μ g) prepared from several peripheral tissues (data not shown). But using conditions in which the SKR6 message is readily detected in rat brain RNAs, we saw no hybridizing signal in rat heart, liver, kidney, spleen, thymus, small intestine, testes and ovary RNAs. These data do not prove the absence of cannabinoid receptors in these tissues as they may be present at considerably lower abundance than in brain. *b*, Low-magnification photograph of an *in situ* hybridization histochemical autoradiogram. In this negative image of a coronal rat brain section, the silver grains appear white. Very high levels of SKR6 mRNA are expressed in isolated cells of the hippocampus and cerebral cortex. In the hippocampus, the strongly labelled cells include granule cells in the dentate gyrus (arrow) as well as cells in both the pyramidal and molecular layers of Ammon's horn. Similarly, in the cortex, layers II, V and VI contain a moderate number of cells expressing very high levels of SKR6 mRNA. These layers also appear to contain many cells that have a much lower message level. Control sections (hybridized under the same condition with a 48-base probe that corresponds to no known message and that gives no signal on northern blots) give a low level, uniform signal (not shown). Cx, cerebral cortex; Hi, hippocampal formation; VMH, ventromedial hypothalamic nucleus; A, amygdaloid nuclei. *c*, Bright-field photomicrograph of the hilar region (see arrow in *b*) of the dentate gyrus ($\times$ 250). Three heavily labelled cells are shown lining the innermost edge of the granule cell layer of the external limb. An additional five cells with high levels of SKR6 mRNA are associated with the internal limb. *d*, Bright-field photomicrograph of the superficial layers of the cerebral cortex ($\times$ 300) showing cells expressing high levels (arrows) of SKR6 mRNA. In this same brain region, cells that express less message are readily seen when a dark-field condenser is used (image similar to that seen in *b*); these less intensely labelled cells, however, are not easily discernible in bright-field photomicrographs.

METHODS. Northern analysis: RNAs were isolated from cultured cells using the guanidinium thiocyanate method as described previously²⁵ and loaded (10 μ g per lane) into a 1% agarose-formaldehyde gel. After electrophoresis and electrotransfer the filter was hybridized to a nick-translated EcoRV-XbaI fragment (bases 97–1,271) of the SKR6 cDNA, washed (0.1 $\times$ SSPE buffer, 0.1% sodium dodecyl sulphate (SDS), 60 $^{\circ}$ C) and exposed to X-ray-sensitive film for 6 days (-80° C). *In situ* hybridization histochemistry: the brain from a male, Sprague-Dawley rat (200–250 g) was sectioned and the 12- μ m slices were thaw-mounted to gelatin-coated slides. *In situ* hybridization histochemistry was as described previously^{26,27}. An ³⁵S-labelled 48-base oligonucleotide (SKR6-1, complimentary to bases 349–396) was used to probe the section. Under similar hybridization conditions, this oligonucleotide probe hybridized to a single $\sim$ 6 kb band in preparations of rat cerebral cortex, hippocampal and cerebellar RNA (data not shown). Similar hybridization patterns were also observed in brain sections hybridized with another 48-base probe SKR6-2 (complementary to bases 4–51, data not shown);



b was produced by placing this section against X-ray-sensitive film (25 $^{\circ}$ C) for 16 days. The hybridized sections were then dipped in NBT-2 emulsion (Kodak), exposed for 21 or 28 days (4 $^{\circ}$ C), developed, stained with 0.1% toluidine blue and a coverslip applied, to produce the images shown in *c* and *d*.

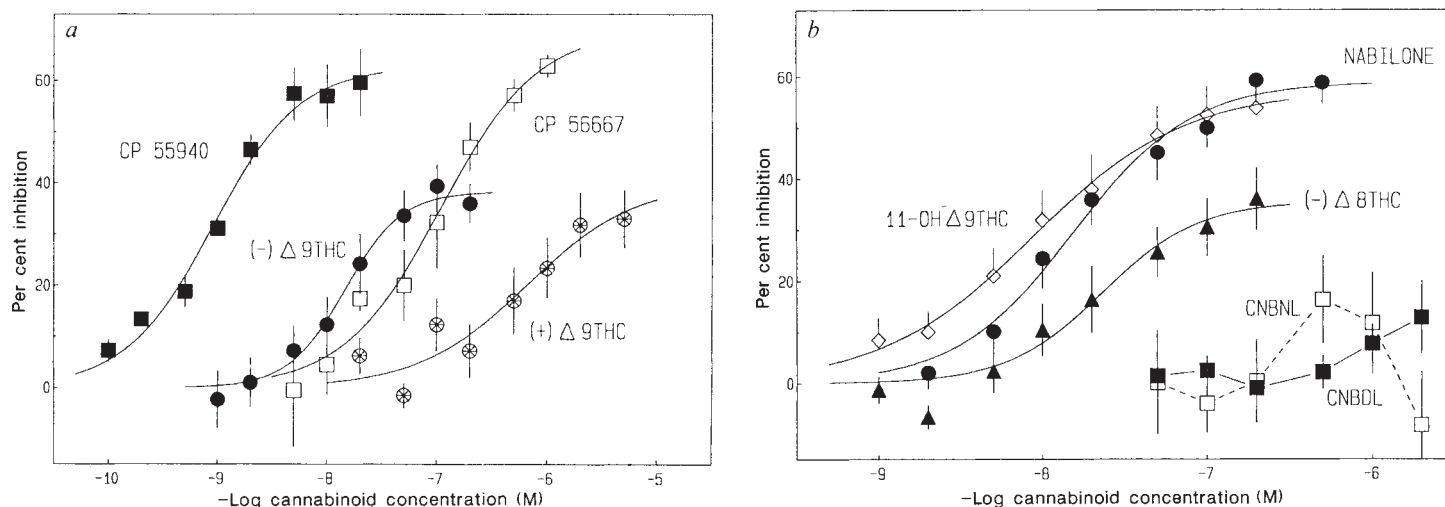


FIG. 3 Cannabinoid-induced inhibition of forskolin-stimulated cAMP production in SKR6-transfected CHO-K1 cells. *a*, Stereoselective inhibition by Δ^9 -THC and CP 55940. *b*, Dose-response curves of various cannabinoids and cannabinoid analogues. Data represent the average per cent inhibition $\pm$ s.e.m. of cAMP accumulation for three to five experiments, each performed in triplicate. Curves were generated using the Graph-Pad InPlot nonlinear regression analysis programme. Cannabinoids did not significantly inhibit cAMP accumulation in nontransfected cells (data not shown). EC_{50} values (mean $nM \pm$ s.e.m.) for the inhibition of stimulated cAMP accumulation were: 13.5 ± 2.7 , $(-)\Delta^9$ -THC; 773 ± 187 , $(+)\Delta^9$ -THC; 0.87 ± 0.20 , CP 55940; 96.3 ± 7.1 , CP 56667; 8.9 ± 1.8 , 11 -OH Δ^9 -THC; 16.6 ± 4.9 , nabilone; 27.4 ± 8.4 , Δ^8 -THC. Cannabinoid-induced inhibition of cAMP accumulation was also observed in transfected cells in which cAMP production was stimulated by the peptide hormone, calcitonin gene-related peptide, instead of forskolin. CP 55940 and CP 56667 are synthesized by Pfizer. Nabilone is produced by Lilly Research laboratories. Other cannabinoids are distributed by the National Institute of Drug Abuse. CNBNL, cannabinol; CNBDL, cannabidiol. METHODS. Transfection and selection of cells were performed as described previously²⁸. A monoclonal line expressing the SKR6 cDNA was obtained by

limiting-dilution cloning of cells expressing the corresponding mRNA as determined by northern blot analysis. Methods used for measurements of cAMP were similar to those of Howlett *et al.*⁶. Transfected cells were grown to confluence and released with 0.5 mM EDTA in PBS. Washed cells were resuspended (1.25×10^6 cells ml^{-1}) in culture media (37 °C) containing HEPES buffer (20 mM) and RO-20 1724 (0.25 mM). Cells were aliquoted (0.4 ml) into silanized glass tubes and the assay initiated with the addition (0.1 ml) of forskolin (0.1 ml, 0.5 μM , final) $\pm$ cannabinoids in media containing fatty acid-free BSA (0.25%). Final ethanol concentrations were less than or equal to 0.2%. Cells were incubated (37 °C) for 5 min and the reaction terminated with the addition of 0.1 N HCl, 0.1 mM $CaCl_2$. Samples were frozen at -20 °C and thawed just before determination of cAMP by radioimmunoassay (refs 29, 30). Forskolin increased cAMP ~ 20 -fold above basal concentrations; absolute values in forskolin-stimulated controls ranged from 9.5 to 17.7 pmole cAMP per 10^6 cells per 5 min. In experiments involving pertussis toxin, confluent cultures of cells were grown in the presence of the holoenzyme (1 ng ml^{-1}) for 24 hours before treatment with forskolin $\pm$ cannabinoids.

between the relative amounts of SKR6 mRNA and cannabinoid receptors¹² in individual brain areas is substantial. High levels of both SKR6 message and cannabinoid receptors (localized by ³H-labelled CP 55940 autoradiography; ref. 12) are found in the dentate gyrus, hippocampal formation and the cerebral cortex (Fig. 2b, and ref. 12). A striking feature of the SKR6 message, in these areas, is the presence of many isolated cells expressing very high levels of receptor message (Fig. 2c, d). Assuming that protein expression is proportional to message levels, these cells probably account for the very high density of cannabinoid receptor reported previously¹². Although more diffuse, there were moderate to high amounts of message in the hypothalamus and amygdala. Although receptors in these regions are relatively

sparsely distributed¹², these data support the notion that cannabinoid-induced effects in the brain are mediated by the same receptor as found in neural cell lines and in cell lines expressing the SKR6 cDNA.

Our data do not eliminate the possibility that other mechanisms also contribute to various cannabinoid-induced effects. Assuming there is an endogenous 'cannabinoid,' SKR6-transfected cell lines can be used to facilitate its identification and purification. These cell lines should prove particularly valuable, as an antagonist for this receptor is not so far available. Addressing the physiological significance of both this receptor and its endogenous ligand should increase our understanding of not only the actions of the cannabinoids but also the CNS. $\square$

Received 24 April; accepted 8 June 1990.

- Hollister, L. E. *Pharmac. Rev.* **38**, 1-20 (1986).
- Martin, B. R. *Pharmac. Rev.* **38**, 45-74 (1986).
- Dewey, W. L. *Pharmac. Rev.* **38**, 151-178 (1986).
- Howlett, A. C. *Molec. Pharmacol.* **27**, 429-436 (1985).
- Howlett, A. C. & Fleming, R. M. *Molec. Pharmacol.* **26**, 532-538 (1984).
- Howlett, A. C., Quail, J. M. & Khachatrian, L. L. *Molec. Pharmacol.* **29**, 307-313 (1986).
- Devane, W. A., Dysarz, F. A., Johnson, M. R., Melvin, L. S. & Howlett, A. C. *Molec. Pharmacol.* **34**, 605-613 (1988).
- Howlett, A. C. *Neuropharmacology* **26**, 507-512 (1987).
- Bonner, T. I., Buckley, N. J., Young, A. C. & Brann, M. R. *Science* **237**, 527-532 (1987).
- Nirenberg, M. *et al. Science* **222**, 794-799 (1983).
- Devane, W. A., Spain, J. W., Coscia, C. J. & Howlett, A. C. *J. Neurochem.* **46**, 1925-1935 (1986).
- Herkenham, M. *et al. Proc. natn. Acad. Sci. U.S.A.* **87**, 1932-1936 (1990).
- Bidaut-Russell, M., Devane, W. A. & Howlett, A. C. *J. Neurochem.* **55**, 21-26 (1990).
- Howlett, A. C., Johnson, M. R., Melvin, L. S. & Milne, G. M. *Molec. Pharmacol.* **33**, 297-302 (1988).
- Razdan, R. K. *Pharmac. Rev.* **38**, 75-149 (1986).
- Gilman, A. G. *Cell* **36**, 577-579 (1984).
- Lawrence, D. K. & Gill, E. W. *Molec. Pharmacol.* **11**, 595-602 (1975).
- Hillard, C. J., Harris, R. A. & Bloom, A. S. *J. Pharmac. exp. Ther.* **232**, 579-588 (1985).
- Masu, Y. *et al. Nature* **329**, 836-838 (1987).

- McFarland, K. C. *et al. Science* **245**, 494-499 (1989).
- Loosfelt, H. *et al. Science* **245**, 525-528 (1989).
- Young, W. S., Waitches, G., Birchmeier, C., Fasano, O. & Wigler, M. *Cell* **45**, 711-719 (1986).
- Karnik, S. S., Sakmar, T. P., Chen, H.-B. & Khorana, H. G. *Proc. natn. Acad. Sci. U.S.A.* **85**, 8459-8463 (1988).
- Okayama, H. & Berg, P. *Molec. cell. Biol.* **3**, 280-289 (1983).
- Chomczynski, P. & Sacchi, N. *Analyt. Biochem.* **162**, 156-159 (1987).
- Young, W. S., Bonner, T. I. & Brann, M. R. *Proc. natn. Acad. Sci. U.S.A.* **83**, 9827-9831 (1986).
- Young, W. S., Warden, M. & Mezey, E. *Neuroendocrinology* **46**, 439-444 (1987).
- Chen, C. & Okayama, H. *Molec. cell. Biol.* **7**, 2745-2752 (1987).
- Brooker, G., Harper, J. F., Terasaki, W. L. & Moylan, R. D. *Adv. Cyclic Nucleotide Res.* **10**, 1-33 (1979).
- Borst, S. & Conolly, M. *Life Sci.* **43**, 1021-1030 (1988).

ACKNOWLEDGEMENTS. We thank H. Okayama for the pCNeo plasmid and M. Nirenberg for cell lines N18TG-2 and C6BU-1. Special thanks to M. Herkenham for providing samples of various cannabinoids and for disclosing autoradiographic data before publication, K. Kusano for his assistance in screening ligands in the *Xenopus* oocyte expression studies and to C. Gerfen for production of photographs. We also thank F. Gusovsky, R. Karier and C. Felder for insightful discussions and technical guidance. L.A.M. was sponsored by the National Institutes of General Medical Sciences as a Pharmacological Research Associate Training fellow. S.J.L. was supported by a C. J. Martin Fellowship from the National Health and Medical Research Council of Australia and awards from the McKnight and Stanley Foundations.

Channel kinetics determine the time course of NMDA receptor-mediated synaptic currents

Robin A. J. Lester, John D. Clements,
Gary L. Westbrook & Craig E. Jahr

The Vollum Institute, Oregon Health Sciences University, Portland,
Oregon 97201, USA

SYNAPTIC release of glutamate results in a two component excitatory postsynaptic current (e.p.s.c.) at many vertebrate central synapses¹⁻⁶. Non-N-methyl-D-aspartate receptors mediate a component that has a rapid onset and decay while the component mediated by N-methyl-D-aspartate (NMDA) receptors has a slow rise-time and a decay of several hundred milliseconds, 100 times longer than the mean open time of NMDA channels⁷⁻⁹. The slow decay of the NMDA-mediated e.p.s.c. could be due to residual glutamate in the synaptic cleft resulting in repeated binding and activation of NMDA receptors. However, in cultured hippocampal neurons, we find that the NMDA receptor antagonist D-2-amino-5-phosphonopentanoate has no effect on the slow e.p.s.c. when rapidly applied after activation of the synapse, suggesting that rebinding of glutamate does not occur. In addition, a brief pulse of glutamate to an outside-out membrane patch results in openings of NMDA channels that persist for hundreds of milliseconds, indicating that glutamate can remain bound for this period. These results imply that a brief pulse of glutamate in the synaptic cleft is sufficient to account for the slow e.p.s.c.

Whole cell patch-clamp techniques were used to record simultaneously from two neurons in hippocampal cultures. Stimulation of an action potential in one neuron evoked an e.p.s.c. in the second neuron that consisted of two distinct components (Fig. 1a). The fast component of the e.p.s.c. was blocked by the addition of the quisqualate/kainate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione^{2,6,10-12} (CNQX, 2.5 μ M) whereas the slow component was blocked by either D-2-amino-5-phosphonopentanoate^{1-6,11-13} (AP5, 20 μ M) or, at hyperpolarized potentials, by extracellular magnesium ions^{1,5,6,12,13}. If transmitter rebinding underlies the long duration of the NMDA receptor-mediated e.p.s.c., the synaptic response should be curtailed by competitive NMDA receptor antagonists when applied during the decay of the synaptic current. To address this possibility, rapid solution changes were made during the slow e.p.s.c. by switching between control and drug-containing superfusate flowing from adjacent flow pipes¹⁴ (Fig. 1b). As Mg^{2+} rapidly blocks open NMDA channels^{7,15}, we used it to test the speed of solution change. Mg^{2+} (100 μ M) blocked the slow e.p.s.c. with a time constant of 29.5 ± 5.4 ms (mean $\pm$ s.e.m., $n = 8$; Fig. 1c), much faster than the decay of the e.p.s.c. A submaximal concentration of Mg^{2+} was used so that the time-course of the inhibition should closely mimic the rise in Mg^{2+} concentration in the synaptic cleft. The inhibition of the e.p.s.c. remained coincident with the switch of solutions as the interval between presynaptic stimulation and Mg^{2+} application was decreased (Fig. 1c).

By contrast to Mg^{2+} , when AP5 (100 μ M) reached the synapse after it was activated, the slow e.p.s.c. was unaffected (Fig. 1d). Blockade of the slow e.p.s.c. by AP5 only occurred when the application preceded the presynaptic stimulus by more than the solution-exchange time (Fig. 1d, last trace). As AP5, even at supramaximal concentrations, had no effect when it was applied after synaptic activation, rebinding of glutamate must not occur during most of the slow e.p.s.c. Rather, NMDA receptors activated by synaptically released glutamate must remain bound for the duration of the slow e.p.s.c. and thus, inaccessible to competitive inhibition.

To determine the underlying channel kinetics, brief pulses of

glutamate were applied to outside-out membrane patches from hippocampal neurons by rapidly moving the interface of two flowing solutions across the tip of the patch pipette (Fig. 2a). Pulses of glutamate to outside-out patches resulted in channel activity that outlasted the glutamate application by hundreds of milliseconds (Fig. 2b). The single-channel currents were due to NMDA channel activity, as they were not blocked by CNQX (2 μ M) and had amplitudes consistent with a conductance of 40–50 pS⁷⁻⁹. When the glutamate pulse was terminated by switching into a submaximal concentration of Mg^{2+} (100 μ M), the sustained channel activity was instantaneously changed from well-resolved openings to brief flickers, due to the rapid channel block by Mg^{2+} ^{7,15} (Fig. 2c). The ensemble-average of currents resulting from repeated applications, show that solution exchange was complete in 1 ms (Fig. 2c). When the glutamate application was terminated by switching into 100 μ M AP5, however, the decay of channel activity (Fig. 2d) was similar to that seen in the absence of AP5 (Fig. 2b). In contrast to the results with Mg^{2+} , ensemble-averages of trials terminated by AP5 displayed no rapid block of the current at the time of

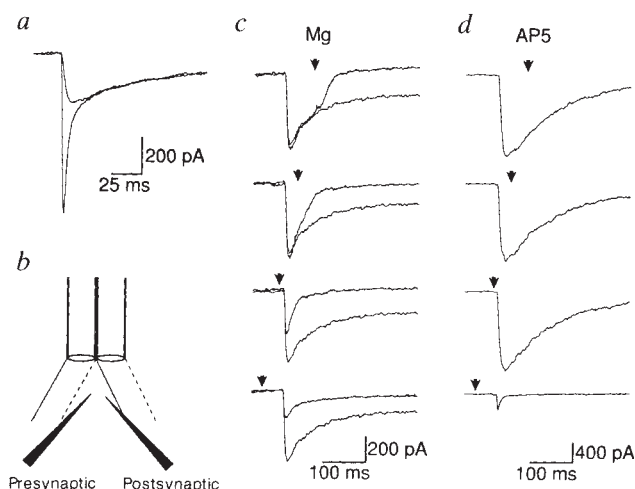


FIG. 1 Rebinding of transmitter does not contribute to the duration of the NMDA receptor-mediated e.p.s.c. *a*, Superimposed records of e.p.s.c.s recorded in control medium and the presence of 2.5 μ M CNQX that selectively blocked the fast component (each trace is an average of 10 responses). *b*, Diagram of the flow pipe apparatus used to switch the superfusate in the synaptic field. Solution changes were effected by closing the flow pipe containing control medium 20 ms before the adjacent drug-containing pipe was opened. Medium from only one pipe was flowing at a time. *c*, The flow pipe containing Mg^{2+} was opened for 400 ms at 140, 100, 60 and 20 ms (arrows) after the beginning of each trial as determined by tip potential changes measured after the experiment. The presynaptic neuron was always stimulated at 60 ms. Individual trials were separated by 5 s. The e.p.s.c.s recorded in control medium and during Mg^{2+} application are superimposed. *d*, The same paradigm as in *c* was used except AP5 (100 μ M) was substituted for Mg^{2+} . Traces in *c* and *d* were recorded in the presence of 5 μ M CNQX and are each an average of 3–5 trials.

METHODS. Hippocampi from 1–3-day-old rats were enzymatically and mechanically dissociated and grown in long-term cell culture as previously described^{1,23}. Whole-cell recordings from two cells were obtained using patch-clamp amplifiers (Warner Instruments). Currents were digitally sampled at 2 kHz and low-pass filtered at 1 kHz. Pipettes contained K gluconate, 150 mM; NaCl, 10 mM; HEPES buffer, 10 mM; EGTA, 10 mM. Control extracellular solution contained NaCl, 155 mM; KCl, 3 mM; $CaCl_2$, 2 mM; HEPES buffer, 10 mM; D-glucose, 10 mM; glycine, 0.02 mM; picrotoxin, 0.05 mM (pH 7.4). Reservoirs of media were connected to a series of glass flow pipes (400 μ m internal diameter) by solenoid latching valves (General Valve) and were activated remotely by computer. The pipes were tilted downwards at about 30° to horizontal and positioned to within 200 μ m. Flow rate at the tip of each pipe was 8 cm s⁻¹.

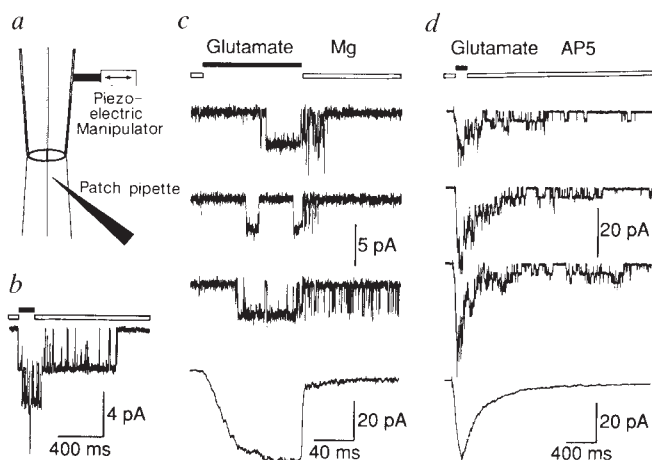


FIG. 2 NMDA channel activity in outside-out patches outlasts glutamate application. *a*, Diagram of superfusion system. Solution changes were effected by moving the interface of solutions flowing from adjacent barrels of θ -tubing across the tip of the patch pipette (see methods). *b*, Channel activity resulting from a 100 ms pulse of 100 μ M glutamate (indicated by step in top trace). Ensemble-averages from 6 patches were fitted with double exponential functions with mean time-constants of 76 ± 2 and 548 ± 65 ms. *c*, Examples of channel activity resulting from 100 ms pulse of 100 μ M glutamate terminated by switching into 100 μ M Mg^{2+} (top 3 traces). Ensemble-average of 63 trials in a different patch (bottom trace). *d*, Same as in *c* except that 100 μ M AP5 was substituted for Mg^{2+} (130 trials). Double exponential time-constants were 76 ± 6 and 390 ± 38 ($n=4$).

METHODS. Currents were recorded (Axopatch 1C) from outside-out patches taken from hippocampal neurons and were digitally sampled at 1 kHz and low-pass filtered at 500 Hz, except for those illustrated in Fig. 2*b*, which were sampled and filtered at 10 and 5 kHz, respectively. External solutions were gravity fed to each lumen of glass θ -tubing pulled to a final tip diameter of 180 μ m (septum thickness ~ 20 μ m). Both solutions were constantly flowing and, within 50–100 μ m of the tip where the patch pipette was positioned, a sharp interface formed between the solutions. Solution changes were effected by rapidly moving the interface of the solutions across the tip of the patch pipette using a piezoelectric translator (Physik Instrumente model P245.30). Control external solution contained NaCl, 180 mM; KCl, 2.5 mM; $CaCl_2$, 1 mM; HEPES buffer, 10 mM; glycine, 0.01 mM; picrotoxin, 0.02 mM; CNQX, 0.002 mM; tetrodotoxin 0.0008 mM (pH 7.3). Internal solution contained CsCl, 150 mM; HEPES buffer, 10 mM; EGTA, 10 mM (pH 7.2).

switching (Fig. 2*d*). These data are consistent with the results of the synaptic experiments and support the suggestion that glutamate remains bound to NMDA receptors for prolonged periods.

Ensemble-averages of patch currents evoked by brief pulses of glutamate had strikingly similar time courses to NMDA e.p.s.c.s. The decay phases of responses to synaptic stimulation or a 5 ms glutamate pulse (Fig. 3) were fitted by double exponentials. The first time constant was 63 ± 3 ms for the e.p.s.c. ($n=7$) and 87 ± 5 ms for the ensemble averages ($n=8$). The values of the long time constants were more variable, ranging 250–545 ms for the e.p.s.c. and 260–600 ms for the patch response. The presence of long components in both responses is consistent with a kinetic scheme that includes a long-lasting closed or desensitized. The 10–90% rise-times of e.p.s.c.s and ensemble-averages of patch currents were 7.4 ± 2.4 ($n=18$) and 10.3 ± 3.2 ms ($n=13$), respectively (Fig. 3, insets). As the rise-time of ensemble patch currents was significantly longer than the period during which glutamate was present, this suggests that the slow rise-time of the e.p.s.c. is due to the activation kinetics of NMDA receptors and does not require diffusion of transmitter to extra-synaptic sites.

The slow activation of NMDA channels suggests several closed states between agonist binding and the open state,

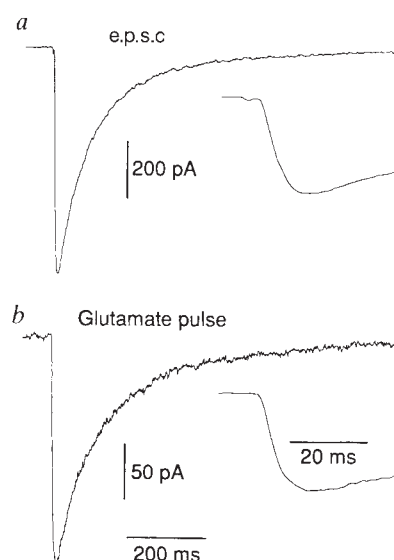


FIG. 3 The time-course of patch currents evoked by brief pulses of glutamate mimicked the time course of NMDA e.p.s.c.s. *a*, An average of 30 e.p.s.c.s and *b*, an ensemble-average of 80 responses of an outside-out patch resulting from a 5-ms application of glutamate (100 μ M) both in the presence of CNQX. The decay phases of both are well fitted with a double exponential with a time constants of 71 and 272 ms for the e.p.s.c. and 83 and 323 ms for the patch response, respectively. Insets compare the rise-times of the two responses at a faster time base. The 10–90% rise-times of the e.p.s.c. and the patch response were 7.7 and 7.9 ms, respectively.

whereas the long duration is due to prolonged occupation of the receptor by glutamate. The resulting 'bursts' of NMDA channel activity have different origins from those of the endplate nicotinic receptor in that the latter are due to the rapid association and dissociation of acetylcholine¹⁶ rather than transitions between open and closed (but bound) states. This reflects the almost 100-fold difference in the affinities of the receptors for their agonists; 1 μ M glutamate^{17,18} for the NMDA receptor and 77 μ M acetylcholine for the nicotinic receptor¹⁶. As at the endplate¹⁹, if ligands with lower affinity for the NMDA receptor (such as aspartate) were released synaptically, it would be expected that shorter duration e.p.s.c.s would result. This agonist-specific difference could alter synaptic efficacy depending on whether aspartate or glutamate is released.

Although our experiments do not directly address the lifetime of free transmitter in the cleft, the results indicate that a brief pulse of transmitter is all that is necessary for prolonged activation of NMDA receptors. Because of slow unbinding, NMDA channel activity will continue in the absence of free transmitter. Rapid clearance of transmitter by diffusion and re-uptake has important functional implications at excitatory synapses. In particular, it would prevent the desensitization of non-NMDA receptors^{20,21} and presynaptic depression²² that would result from tonic glutamate in the synaptic cleft. □

Received 25 April; accepted 17 May 1990.

1. Forsythe, I. D. & Westbrook, G. L. *J. Physiol., Lond.* **396**, 515–533 (1988).
2. Westbrook, G. L. & Jahr, C. E. *Seminars in the Neurosciences* **1**, 103–114 (1989).
3. Dale, N. & Roberts, A. *J. Physiol., Lond.* **363**, 35–59 (1985).
4. Bekkers, J. M. & Stevens, C. F. *Nature* **341**, 230–233 (1989).
5. Collingridge, G. L., Herron, C. E. & Lester, R. A. *J. Physiol., Lond.* **399**, 283–300 (1988).
6. Hestrin, S., Nicoll, R. A., Perkel, D. J. & Sah, P. *J. Physiol., Lond.* **422**, 203–225 (1990).
7. Nowak, L., Bregestovski, P., Ascher, P., Herbet, A. & Prochiantz, A. *Nature* **307**, 462–465 (1984).
8. Jahr, C. E. & Stevens, C. F. *Nature* **325**, 522–525 (1987).
9. Cull-Candy, S. G. & Usowicz, M. M. *Nature* **325**, 525–528 (1987).
10. Blake, J. F., Brown, M. W. & Collingridge, G. L. *Neurosci. Lett.* **89**, 182–186 (1988).
11. Andreasen, M., Lambert, J. D. C. & Jensen, M. S. *J. Physiol., Lond.* **414**, 317–336 (1989).
12. Kauer, J. A., Malenka, R. C. & Nicoll, R. A. *Neuron* **1**, 911–917 (1988).
13. Wigstrom, H., Gustafsson, B. & Huang, Y.-Y. *Acta physiol. scand.* **124**, 475–478 (1985).
14. Sernagor, E., Kuhn, D., Vyklícký, L. & Mayer, M. *Neuron* **2**, 1221–1227 (1989).
15. Mayer, M. L., Westbrook, G. L. & Guthrie, P. B. *Nature* **309**, 261–263 (1984).

16. Colquhoun, D. & Ogden, D. C. *J. Physiol., Lond.* **395**, 131–159 (1988).
17. Patneau, D. K. & Mayer, M. L. *J. Neurosci.* (in the press).
18. Olverman, H. J., Jones, A. W. & Watkins, J. C. *Nature* **307**, 460–462 (1984).
19. Colquhoun, D., Large, W. A. & Rang, H. P. *J. Physiol., Lond.* **266**, 361–395 (1977).
20. Trussell, L. O. & Fischbach, G. D. *Neuron* **3**, 209–218 (1989).
21. Tang, C. M., Dichter, M. & Morad, M. *Science* **243**, 1474–1477 (1989).
22. Forsythe, I. D. & Clements, J. D. *J. Physiol., Lond.* (in the press).
23. Lester, R. A. J., Quarum, M. L., Parker, J. D., Weber, E. & Jahr, C. E. *Molec. Pharmac.* **35**, 565–570 (1989).

ACKNOWLEDGEMENTS. This work was supported by the NIH and the McKnight Endowment Fund for Neuroscience (C.E.J. and G.L.W.).

Regions of the skeletal muscle dihydropyridine receptor critical for excitation–contraction coupling

Tsutomu Tanabe*, Kurt G. Beam†‡, Brett A. Adams†, Tetsuhiro Niidome* & Shosaku Numa*‡

* Departments of Medical Chemistry and Molecular Genetics, Kyoto University Faculty of Medicine, Kyoto 606, Japan

† Department of Physiology, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, Colorado 80523, USA

It is thought that in skeletal muscle excitation–contraction (EC) coupling, the release of Ca^{2+} from the sarcoplasmic reticulum is controlled by the dihydropyridine (DHP) receptor in the transverse tubular membrane, where it serves as the voltage sensor^{1–3}. We have shown previously⁴ that injection of an expression plasmid carrying the skeletal muscle DHP receptor complementary DNA³ restores EC coupling and L-type calcium current that are missing in skeletal muscle myotubes from mutant mice with muscular dysgenesis^{5–9}. This restored coupling resembles normal skeletal muscle EC coupling⁴, which does not require entry of extracellular Ca^{2+} (refs 10, 11). By contrast, injection into dysgenic myotubes of an expression plasmid carrying the cardiac DHP receptor cDNA¹² produces L-type calcium current and cardiac-type EC coupling¹³, which does require entry of extracellular Ca^{2+} (refs 14–16). To identify the regions responsible for this important functional difference between the two structurally similar DHP receptors, we have expressed various chimaeric DHP receptor cDNAs in dysgenic myotubes. The results obtained indicate that the putative cytoplasmic region between repeats II and III of the skeletal muscle DHP receptor³ is an important determinant of skeletal-type EC coupling.

The main differences in primary structure between the skeletal muscle³ and cardiac¹² DHP receptors reside in the large, putative cytoplasmic regions, that is, the amino- and carboxy-terminal regions as well as the regions linking repeats I and II (I–II loop) and repeats II and III (II–III loop). To test whether these regions of the skeletal muscle DHP receptor are required for skeletal-type EC coupling, we constructed five different expression plasmids carrying chimaeric DHP receptor cDNAs (Fig. 1). These constructs encode the cardiac DHP receptor as the basic structure in which the large, putative cytoplasmic regions are replaced by the corresponding regions of the skeletal muscle DHP receptor. The cardiac DHP receptor was chosen as the starting structure because the L-type calcium current expressed in myotubes injected with the entirely cardiac DHP receptor cDNA (pCARD1) is about sixfold larger than that expressed in myotubes injected with the entirely skeletal muscle DHP receptor cDNA (pCAC6)¹³. A large L-type current is helpful in identifying cells expressing a chimaeric DHP receptor that fails to mediate skeletal-type EC coupling.

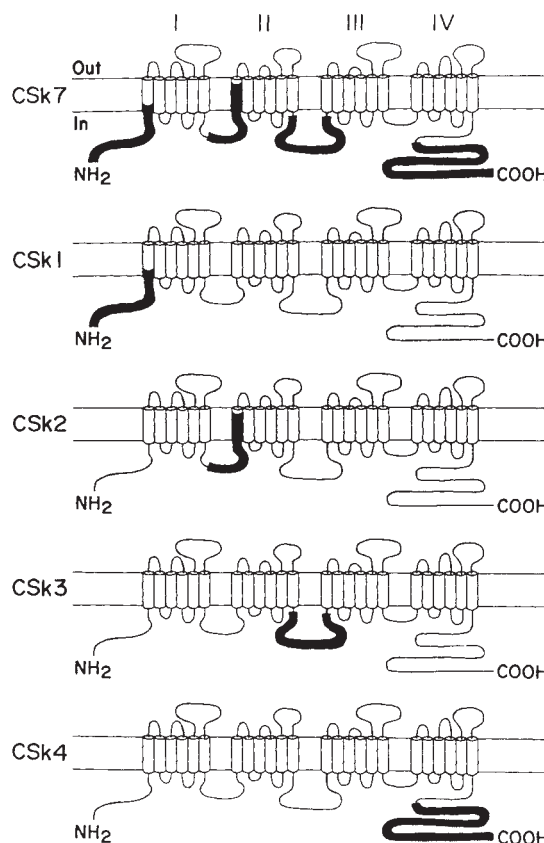


FIG. 1 Schematic representation of the structures of chimaeric DHP receptors (CSk7 and CSk1–4 from top to bottom) composed of cardiac and skeletal muscle sequences. For each chimaeric DHP receptor, the four units of homology (repeats I–IV) are displayed linearly and the six putative transmembrane segments (S1 to S6 from left to right) in each repeat are shown by cylinders. The darkly shaded areas indicate the regions of the cardiac DHP receptor that have been replaced by the corresponding portions of the skeletal muscle DHP receptor. Note that the junctional sequences common to the two DHP receptors are not darkly shaded and that the exchanged portion of segment S1 of repeat II is very similar in amino acid sequence between the two DHP receptors (see below). The compositions of the individual chimaeric DHP receptors are as follows (C and Sk, cardiac and skeletal muscle DHP receptor, respectively; numbers in parentheses, amino-acid numbers^{3,12}; the junctional sequences common to the two DHP receptors are represented by amino-acid numbers of the cardiac DHP receptor). CSk7: Sk(1–55), C(159–464), Sk(364–448), C(571–787), Sk(666–791), C(923–1,634) and Sk(1,510–1,873). CSk1: Sk(1–55) and C(159–2,171). CSk2: C(1–464), Sk(364–448) and C(571–2,171). CSk3: C(1–787), Sk(666–791) and C(923–2,171). CSk4: C(1–1,634) and Sk(1,510–1,873).

METHODS. The expression plasmids (pCSk7 and pCSk1–4) carrying the cDNAs encoding the individual chimaeric DHP receptors were constructed by inserting the corresponding cDNAs into the *Hind*III site of the plasmid pKCRH2 (ref. 17). The chimaeric DHP receptor cDNAs are composed of the following restriction fragments derived from the plasmids pCAC6 (ref. 4) and pCARD1 (ref. 12) (C and Sk in parentheses denote the origin of the fragments and the restriction endonuclease sites are identified by numbers^{3,12} in parentheses indicating the 5'-terminal nucleotide generated by cleavage). pCSk1: 213-base-pair (bp) *Hind*III (Sk 5'-terminal linker)–*Bst*XI (Sk 196) and 6.4-kilobase-pair (kb) *Bst*XI (C 505)–*Hind*III (C 3'-terminal linker); there is one base difference at the *Bst*XI site between the cardiac and skeletal muscle DHP receptors, which causes no amino-acid difference. pCSk2: 1.6-kb *Hind*III (C 5'-terminal linker)–*Pvu*II (C 1,378), *Pvu*II (Sk 1,075)–*Hgi*AI (Sk 1,359) and 5.2-kb *Hgi*AI (C 1,725)–*Hind*III (C 3'-terminal linker). pCSk3: 2.5-kb *Hind*III (C 5'-terminal linker)–*Xmn*I (C 2,330), *Xmn*I (Sk 1,964)–*Hinc*II (Sk 2,389) and 4.1-kb *Hinc*II (C 2,782)–*Hind*III (C 3'-terminal linker). pCSk4: 5.1-kb *Hind*III (C 5'-terminal linker)–*Bgl*II (C 4,863) and 1.2-kb *Bgl*II (Sk 4,488)–*Hind*III (Sk 3'-terminal linker). pCSk7: 665-bp *Hind*III–*Sac*I from pCSk1, 1.2-kb *Sac*I–*Eco*RI from pCSk2, 1.7-kb *Eco*RI–*Aat*II from pCSk3 and 2.1-kb *Aat*II–*Hind*III from pCSk4.

‡ To whom correspondence should be addressed.

Dysgenic myotubes injected with each of the five chimaeric plasmids (pCSk7 and pCSk1-4) did indeed display electrically evoked contractions; the number of responsive myotubes relative to the number of tested myotubes was comparable to that reported previously¹³ for pCAC6- or pCARD1-injected myotubes. Although not systematically monitored, spontaneous contractions were also observed in some myotubes injected with each of the chimaeric plasmids. Myotubes that had been injected with a chimaeric plasmid and observed to contract all showed L-type calcium current (Fig. 2c-g). The L-type currents induced by the chimaeric plasmids displayed a rapid rate of activation, which was very similar to that in pCARD1-injected myotubes (Fig. 2b) and much faster than that in pCAC6-injected myotubes (Fig. 2a). Two additional similarities were found between the calcium currents produced by the chimaeric plasmids and by pCARD1, as exemplified by a pCSk3-injected myotube (Fig. 3). First, substitution of Ba^{2+} for Ca^{2+} caused an increase in peak current that was comparable to the response of L-type current in myotubes expressing pCARD1 but larger than that in myotubes expressing pCAC6 (ref. 13). Second, with Ca^{2+} as the charge carrier, the potential that activated peak L-type current in myotubes expressing the chimaeric cDNAs or pCARD1 (ref. 13) was 20–40 mV more negative than that in myotubes expressing pCAC6 (refs 4, 13). Thus, properties characteristic of the cardiac L-type channel are maintained even when its major putative cytoplasmic regions are replaced by their skeletal muscle counterparts.

Examination of electrically evoked contractions demonstrated that dysgenic myotubes expressing pCAC6, as reported previously^{4,13}, displayed skeletal-type EC coupling. Thus, for pCAC6-expressing myotubes, contraction was observed both in Ca^{2+} -free Ringer's solution (Fig. 4a, 2) and in 0.5 mM Cd^{2+} -containing Ringer's solution (Fig. 4a, 3) as well as in normal Ringer's (Fig. 4a, 1). In contrast, myotubes expressing

pCARD1 showed cardiac-type EC coupling (Fig. 4b; see also ref. 13). Dysgenic myotubes expressing pCSk7, which encodes the chimaeric DHP receptor with all the major putative cytoplasmic regions replaced by their skeletal muscle counterparts, exhibited EC coupling (Fig. 4c) similar to that produced by pCAC6. Thus, switching these regions of the DHP receptor from cardiac to skeletal muscle changed the character of EC coupling from cardiac-type (Fig. 4b) to skeletal-type (Fig. 4a).

We next examined whether replacement of a single putative cytoplasmic region is sufficient to produce this change. Dysgenic myotubes expressing either pCSk1, which encodes the chimaera with only the amino-terminal region replaced (Fig. 4d), or pCSk4, which encodes the chimaera with only the carboxy-terminal region replaced (Fig. 4g), displayed cardiac-type EC coupling. In contrast, myotubes expressing pCSk3, which encodes the chimaera with only the II-III loop replaced, showed skeletal-type EC coupling (Fig. 4f). Altogether, 42 myotubes expressing pCSk3, as well as 20 expressing pCSk7 and 25 expressing pCAC6, were tested and all were found to display skeletal-type EC coupling. That myotubes expressing pCSk3 or pCSk7 exhibited weaker contraction in Ca^{2+} -free or Cd^{2+} -containing Ringer's than in normal Ringer's (Fig. 4c, f) can be accounted for by entry of extracellular Ca^{2+} contributing to the contraction of these myotubes in normal Ringer's. When tested under identical conditions (extracellularly applied stimulus with a duration of 5 ms), myotubes expressing pCSk2, which encodes the chimaera with only the I-II loop replaced, showed cardiac-type EC coupling. When the stimulus duration was increased to 20 ms, however, weak contractions were observed in some myotubes expressing pCSk2 both in Ca^{2+} -free (9 of 23 tested) and in Cd^{2+} -containing (14 of 20 tested) Ringer's. These weak contractions, barely resolvable by the contraction-monitoring apparatus, are evident as a brief, small, upward deflection at the beginning of the trace (Fig. 4e, 2 and 3). Even

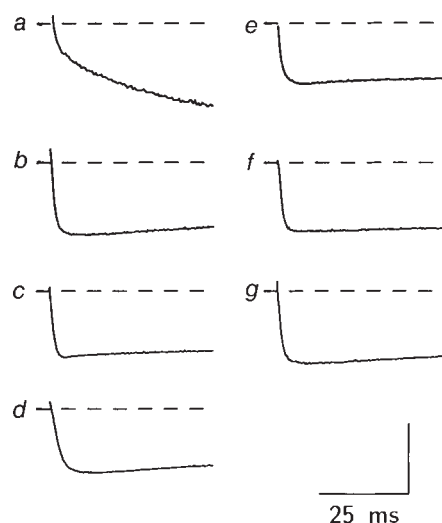


FIG. 2 Comparison of calcium currents in dysgenic myotubes expressing pCAC6 (a), pCARD1 (b), pCSk7 (c), pCSk1 (d), pCSk2 (e), pCSk3 (f) or pCSk4 (g). Calcium currents were measured using the whole-cell variant of the patch-clamp technique¹⁸. For all the currents illustrated, the holding potential was -80 mV and the test potential was $+30$ mV. Vertical calibration corresponds to 1.5 nA (a), 15 nA (b) or 10 nA (c-g). The experimental procedures used were essentially the same as described previously^{4,13}. The linear cell capacitance (C) for each cell was as follows: a, Cell AJ01, $C=500$ pF; b, Cell CA37, $C=450$ pF; c, Cell CA87, $C=335$ pF; d, Cell CB11, $C=490$ pF; e, Cell CA91, $C=565$ pF; f, Cell CA33, $C=250$ pF; g, Cell CA78, $C=560$ pF. The pipette solution contained: 140 mM caesium-aspartate, 5 mM MgCl_2 , 10 mM caesium-EGTA, 10 mM HEPES buffer (pH 7.4 with CsOH). The bathing solution contained: 10 mM Ca^{2+} , 145 mM tetraethylammonium ion, 165 mM Cl^- , 0.003 mM tetrodotoxin, 10 mM HEPES buffer (pH 7.4 with CsOH). Temperature 20–22 °C.

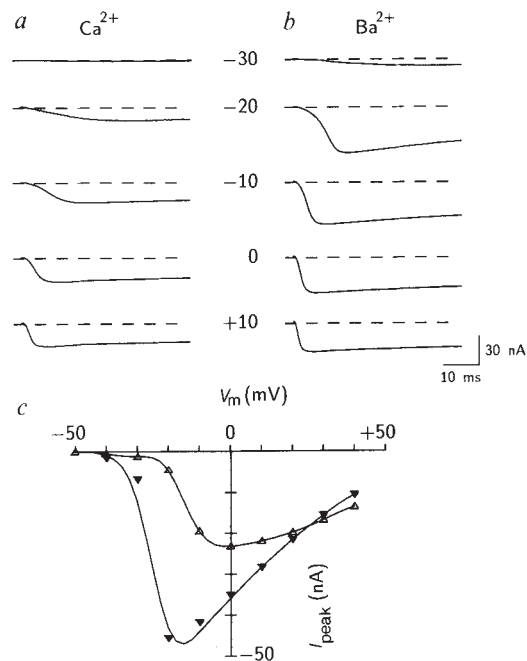


FIG. 3 L-type currents carried by Ca^{2+} and Ba^{2+} in a dysgenic myotube expressing pCSk3. a, b, Currents evoked by stepping from the holding potential (-80 mV) to the test potential V_m (in mV) indicated next to each trace, with either 10 mM Ca^{2+} (a) or 10 mM Ba^{2+} (b) as the charge carrier. c, Peak current (I_{peak}) for these records plotted as a function of test potential (open symbols, Ca^{2+} ; closed symbols, Ba^{2+}). The composition of the bathing solution containing 10 mM Ca^{2+} is described in Fig. 2 legend. The bathing solution containing 10 mM Ba^{2+} was identical, except for equimolar substitution of Ba^{2+} for Ca^{2+} . The data are from cell CA35, $C=780$ pF.

with the longer stimulus of 20-ms duration, myotubes expressing pCSk1 ($n=15$), pCSk4 ($n=13$) or pCARD1 ($n=15$) never showed contractions in Ca^{2+} -free or Cd^{2+} -containing Ringer's.

In addition to using extracellular stimulation, we also examined contraction using the whole-cell patch-clamp technique, where only 0.1 mM EGTA was present in the solution filling the patch-pipette¹³. In Ca^{2+} -free, 10 mM Mg^{2+} -containing bathing solution, stepping from the holding potential (-80 mV) to $+10$ mV for 20 ms caused contraction of all the tested myotubes expressing pCSk3 ($n=9$) or pCAC6 ($n=3$). Under identical test conditions, three of 12 myotubes expressing pCSk2 contracted, but the other nine myotubes did not contract even when the duration of the stimulus was increased to 400 ms. Myotubes expressing pCSk1 ($n=7$), pCSk4 ($n=3$) or pCARD1 ($n=9$) did not contract in the Ca^{2+} -free test solution even with a 400-ms stimulus. In conclusion, pCSk3 produces effective skeletal-type EC coupling. pCSk2 is also able to produce skeletal-type EC coupling, although the efficiency is so low that the amount of Ca^{2+} released from the sarcoplasmic reticulum exceeds the threshold for contraction in only a fraction of cells.

Our results indicate that the II-III loop of the skeletal muscle DHP receptor is a major determinant site for skeletal-type EC coupling, that the I-II loop is possibly less important and that the amino- and carboxy-terminal regions are probably unimpor-

tant. Alternatively, the weaker skeletal-type EC coupling observed with pCSk2 than with pCSk3 may mean that the I-II and the II-III loop are both critical, but that the I-II loop is more functionally interchangeable between the cardiac and skeletal muscle DHP receptors than is the II-III loop. Similarly, it is possible that the putative cytoplasmic region linking repeats III and IV, which is highly conserved between the two DHP receptors, is also important in skeletal-type EC coupling. The finding that expression of pCSk3 and pCSk7 produces skeletal-type EC coupling but rapidly activating, cardiac-type calcium current shows that the slow activation of the skeletal muscle L-type channel is not an obligatory consequence of the DHP receptor serving as the voltage sensor for EC coupling. This finding also suggests that the putative membrane-spanning and adjacent regions of the DHP receptor, forming the channel and its entrances, are important in determining the properties of L-type calcium current. □

Received 30 March; accepted 5 June 1990.

1. Schneider, M. F. & Chandler, W. K. *Nature* **242**, 244-246 (1973).
2. Rios, E. & Brum, G. *Nature* **325**, 717-720 (1987).
3. Tanabe, T. *et al.* *Nature* **328**, 313-318 (1987).
4. Tanabe, T., Beam, K. G., Powell, J. A. & Numa, S. *Nature* **336**, 134-139 (1988).
5. Gluecksohn-Waelsch, S. *Science* **142**, 1269-1276 (1963).
6. Powell, J. A. & Fambrough, D. M. *J. cell. Physiol.* **82**, 21-38 (1973).
7. Klaus, M. M., Scordilis, S. P., Rapalus, J. M., Briggs, R. T. & Powell, J. A. *Dev. Biol.* **99**, 152-166 (1983).
8. Beam, K. G., Knudson, C. M. & Powell, J. A. *Nature* **320**, 168-170 (1986).
9. Rieger, F. *et al.* *Nature* **330**, 563-566 (1987).
10. Armstrong, C. M., Bezanilla, F. M. & Horowicz, P. *Biochim. biophys. Acta* **267**, 605-608 (1972).
11. Knudson, C. M., Jay, S. D. & Beam, K. G. *Biophys. J.* **49**, 13a (1986).
12. Mikami, A. *et al.* *Nature* **340**, 230-233 (1989).
13. Tanabe, T., Mikami, A., Numa, S. & Beam, K. G. *Nature* **344**, 451-453 (1990).
14. Fabiato, A. *J. gen. Physiol.* **85**, 291-320 (1985).
15. Beuckelmann, D. J. & Wier, W. G. *J. Physiol., Lond.* **405**, 233-255 (1988).
16. Nábauer, M., Callewaert, G., Cleemann, L. & Morad, M. *Science* **244**, 800-803 (1989).
17. Mishina, M. *et al.* *Nature* **307**, 604-608 (1984).
18. Hamill, O. P., Marty, A., Neher, E., Sakmann, B. & Sigworth, F. J. *Pflügers Arch. ges. Physiol.* **391**, 85-100 (1981).

ACKNOWLEDGEMENTS. We thank L. VanZuilen and A. Beam for their help in providing cultured myotubes. This research was supported in part by the NIH, the Muscular Dystrophy Association, the Ministry of Education, Science and Culture of Japan, the Mitsubishi Foundation, and the Japanese Foundation of Metabolism and Diseases. B. A. A. was supported by an NIH postdoctoral fellowship.

Intramembrane charge movement restored in dysgenic skeletal muscle by injection of dihydropyridine receptor cDNAs

Brett A. Adams*, Tsutomu Tanabe†, Atsushi Mikami†, Shosaku Numa† & Kurt G. Beam*

* Department of Physiology, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, Colorado 80523, USA

† Departments of Medical Chemistry and Molecular Genetics, Kyoto University Faculty of Medicine, Kyoto 606, Japan

THE skeletal muscle dihydropyridine (DHP) receptor is essential in excitation-contraction (EC) coupling¹⁻⁴. The receptor is postulated to be the voltage sensor giving rise to the intramembrane current, termed charge movement⁵. We have now tested this hypothesis using myotubes from mice with the muscular dysgenesis mutation, which alters the skeletal muscle DHP receptor gene and prevents its expression^{3,4,6}. Our results indicate that charge movement is deficient in dysgenic myotubes but is fully restored following injection of an expression plasmid carrying the rabbit skeletal muscle DHP receptor complementary DNA, strongly supporting the hypothesis that the DHP receptor is the voltage sensor for EC coupling in skeletal muscle. Additionally, our data obtained for normal and chimaeric DHP receptor constructs demonstrate that DHP receptors with widely differing abilities to function as calcium

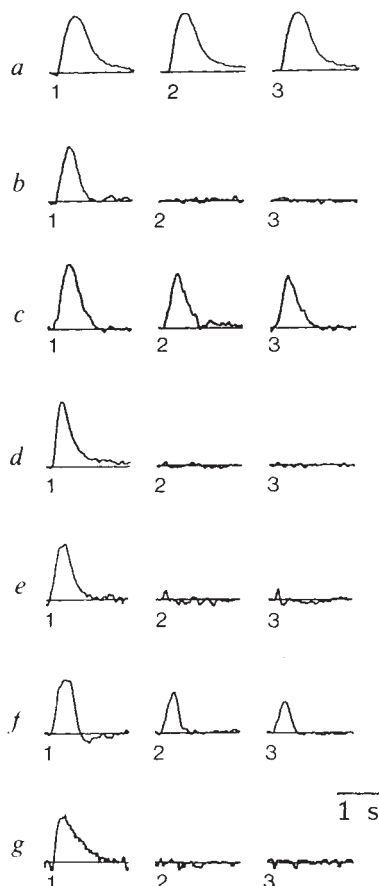


FIG. 4 Comparison of electrically evoked contractions in dysgenic myotubes expressing pCAC6 (a), pCARD1 (b), pCSk7 (c), pCSk1 (d), pCSk2 (e), pCSk3 (f) or pCSk4 (g). Contractions were recorded initially in normal rodent Ringer's (1) and subsequently in a test solution that was either Ca^{2+} -free (2) or 0.5 mM Cd^{2+} -containing (3) Ringer's. A period in normal Ringer's sufficient for full recovery was interspersed between exposures to the two test solutions. The methods for extracellular electrical stimulation and optical recording of contraction were as described previously^{4,13}. The stimulus duration was 5 ms for all the traces, except 20 ms for e, 2 and e, 3. The normal rodent Ringer's contained: 146 mM NaCl, 5 mM KCl, 2 mM CaCl_2 , 1 mM MgCl_2 , 10 mM HEPES buffer (pH 7.4 with NaOH). The Ca^{2+} -free Ringer's was made by equimolar substitution of Mg^{2+} for Ca^{2+} in the normal rodent Ringer's. Horizontal calibration, 1 s. Vertical scale, arbitrary units.

TABLE 1 Expression of immobilization-resistant charge movement and L-type calcium conductance

Myotubes	$Q_{\max}$ (nC μF^{-1})	$\bar{V}_Q$ (mV)	$G_{\max}$ (nS nF $^{-1}$)	$\frac{G_{\max}}{Q'_{\max}}$ (nS pC $^{-1}$)
Dysgenic	2.5 $\pm$ 0.8 (11)	-24 $\pm$ 2.8 (11)	—	—
Normal (S)	7.9 $\pm$ 1.4 (8)	-13 $\pm$ 3.0 (8)	459 $\pm$ 92 (8)	85
pCAC6 (S)	6.4 $\pm$ 2.8 (10)	-13 $\pm$ 4.2 (7)	141 $\pm$ 80 (11)	36
pCARD1(C)	11.4 $\pm$ 6.0 (12)	-13 $\pm$ 2.0 (9)	488 $\pm$ 171 (11)	55
pCsk7 (S)	10.2 $\pm$ 4.4 (9)	-9 $\pm$ 2.1 (5)	274 $\pm$ 137 (11)	36
pCsk1 (C)	15.4 $\pm$ 6.5 (9)	-7 $\pm$ 2.1 (7)	448 $\pm$ 220 (11)	35
pCsk2 (S)*	6.7 $\pm$ 2.8 (8)	-13 $\pm$ 0.0 (3)	179 $\pm$ 62 (8)	43
pCsk3 (S)	6.4 $\pm$ 4.8 (7)	-14 $\pm$ 5.0 (3)	611 $\pm$ 221 (7)	157
pCsk4 (C)	8.5 $\pm$ 4.1 (8)	-12 $\pm$ 3.4 (4)	440 $\pm$ 137 (9)	73

* Weak skeletal-type EC coupling.

Data are given as means $\pm$ s.d.; numbers in parentheses indicate the number of myotubes tested. Letters in parentheses indicate whether EC coupling was skeletal-type (S) or cardiac-type (C); see ref. 8. Values of immobilization-resistant Q_{ON} were determined with the prepulse protocol as described in Fig. 1 methods and were fitted¹⁷ according to $Q_{\text{ON}} = Q_{\max} / \{1 + \exp [-(V - \bar{V}_Q)/k_Q]\}$, where $Q_{\max}$ is the maximum immobilization-resistant charge that can be moved, V is the test potential, $\bar{V}_Q$ is the potential at which half the charge has moved and k_Q is a slope factor. For cells where complete Q_{ON} versus V data were not obtained, $Q_{\max}$ was determined as described in Fig. 1 legend. The values of $Q_{\max}$ given for normal and dysgenic myotubes differ slightly from those described in the text, which were obtained from a separate set of cells. The values of k_Q were 10.0 $\pm$ 0.7 (dysgenic), 9.0 $\pm$ 1.6 (normal), 10.5 $\pm$ 1.0 (pCAC6), 9.9 $\pm$ 0.7 (pCARD1), 9.7 $\pm$ 0.6 (pCsk7), 10.3 $\pm$ 1.6 (pCsk1), 10.0 $\pm$ 2.0 (pCsk2), 8.1 $\pm$ 0.7 (pCsk3) and 11.2 $\pm$ 0.5 mV (pCsk4), where the n values are those given for entries under $\bar{V}_Q$ in the table. Values of $G_{\max}$, the maximal L-type conductance, were obtained by fitting¹⁷ measured currents according to $I = G_{\max}(V - V_{\text{rev}}) / \{1 + \exp [-(V - \bar{V}_G)/k_G]\}$, where I is the peak L-type current activated at test potential V , V_{rev} is the extrapolated reversal potential, $\bar{V}_G$ is the potential for activation of half-maximal conductance, and k_G is a slope factor. $Q'_{\max}$ is the difference between $Q_{\max}$ and $Q_{\text{dys-max}}$ (2.5 nC μF^{-1}).

channels and to mediate EC coupling produce very similar charge movements.

Using a standard pulse protocol in which depolarizing test pulses were administered directly from a holding potential of -80 mV, we found that the maximum quantity of mobile charge ($Q_{\max}$) in dysgenic myotubes (7.6 ± 3.2 nC μF^{-1} ; mean $\pm$ s.d., $n = 15$) was smaller than, but still a surprisingly large fraction

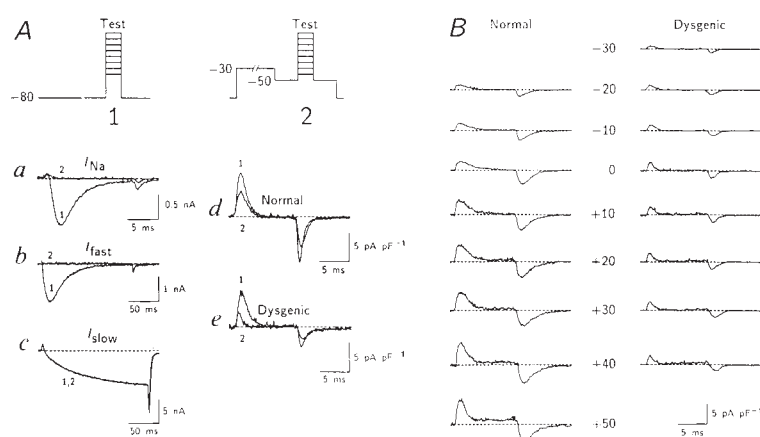
of, that in normal myotubes (12.2 ± 2.9 nC μF^{-1} , $n = 12$). Because some of this charge movement might have arisen from the gating of sodium channels and transient calcium channels, we designed a 'prepulse' protocol (Fig. 1A), which led to complete inactivation of both the sodium current, I_{Na} (Fig. 1A, a), and the transient calcium current, I_{fast} (Fig. 1A, b), without affecting the L-type calcium current, I_{slow} (Fig. 1A, c). Additionally, with the prepulse protocol, part of the charge movement in both normal (Fig. 1A, d) and dysgenic myotubes (Fig. 1A, e) was immobilized. Compared with the standard protocol, $Q_{\max}$ measured with the prepulse protocol was reduced by an average of 4.8 nC μF^{-1} (normal) and 5.4 nC μF^{-1} (dysgenic), the remaining immobilization-resistant charge being 7.4 ± 1.7 ($n = 12$) and 2.2 ± 0.6 nC μF^{-1} ($n = 15$) in normal and dysgenic myotubes, respectively. The comparable reduction of charge movement in normal and dysgenic myotubes is consistent with the idea that the immobilized charge arises from gating of sodium channels and I_{fast} calcium channels, and that these are present at comparable levels in the two kinds of myotubes.

Figure 1B compares the immobilization-resistant charge movement measured at varying test potentials in normal and dysgenic myotubes. In addition to being much smaller, the immobilization-resistant charge in dysgenic myotubes moved over a more negative range of potentials; the potential at which half the charge moved ($\bar{V}_Q$) was shifted about 10 mV negatively compared to normal myotubes (Table 1). This different voltage dependence suggests that the immobilization-resistant charge movement in dysgenic myotubes has a different source from the bulk of that present in normal myotubes. If, as one possibility, potassium channel gating current accounts for the residual charge in dysgenic myotubes, then it would also be expected to contribute a small fraction to the immobilization-resistant charge in normal myotubes.

We next examined whether the immobilization-resistant charge movement deficient in dysgenic myotubes could be restored by injecting them with the expression plasmid pCAC6 encoding the skeletal muscle DHP receptor⁴, pCARD1 encoding the cardiac DHP receptor⁷, or pCsk1-4 and pCsk7 encoding chimaeric DHP receptors⁸. All these expression plasmids indeed

FIG. 1 Reduction of immobilization-resistant charge movement in dysgenic myotubes. A, Ionic currents and charge movements measured with the 'standard' (1) and 'prepulse' (2) protocols are compared. I_{Na} (a) and I_{fast} (b) are completely inactivated with the prepulse protocol, whereas I_{slow} is unaffected (c). With the prepulse protocol, the charge movement signal from normal (d) and dysgenic (e) myotubes is reduced in magnitude. B, Immobilization-resistant charge movement, measured with the prepulse protocol for the indicated test potentials, is compared for normal and dysgenic myotubes. Aa, Cell BH88, dysgenic myotube, linear capacitance (C) = 100 pF, test potential (TP) = -10 mV (this cell lacked I_{fast}). Ab, Cell BH85, dysgenic myotube, $C = 525$ pF, $TP = -30$ mV. Ac, Cell BH93, normal myotube, $C = 570$ pF, $TP = +20$ mV. Ad, Cell BI41, normal myotube, $C = 220$ pF, $TP = +40$ mV. Ae, Cell BI34, dysgenic myotube, $C = 365$ pF, $TP = +30$ mV. B, Cell BI26, $C = 310$ pF, $Q_{\max} = 9.5$ nC μF^{-1} (left panel); cell BH82, $C = 210$ pF, $Q_{\max} = 2.6$ nC μF^{-1} (right panel).

METHODS. The methods for tissue culture and injection of cDNA as well as for measurement of ionic currents and charge movement were essentially as described previously^{3,4,8,13,17}. Test currents were corrected for linear leakage and capacitive currents by digitally scaling and subtracting a control current elicited by a voltage step from -100 to -120 mV. The whole-cell¹⁸ patch pipette contained 140 mM caesium aspartate, 5 mM MgCl₂, 10 mM caesium-EGTA, 10 mM HEPES buffer (pH 7.4 with CsOH). Pipette resistance was minimized (usually to <0.7 M Ω) by electronic compensation. The bath contained 10 mM Ca²⁺, 145 mM tetraethylammonium ion, 165 mM Cl⁻, 0.003 mM tetrodotoxin (TTX), 10 mM HEPES (pH 7.4 with CsOH). Temperature, 20–22 °C. For the measurement of sodium currents, TTX was omitted and 5 mM NaCl was added. For charge movements, 0.5 mM Cd²⁺ and 0.1 mM La³⁺ were added to the bath to block calcium



currents, and the voltage-clamp command pulses were exponentially rounded with a time constant of 50–300 μs . For a given test pulse, Q_{ON} was obtained by integrating the transient of charge that moved outward following the onset of the test pulse, and Q_{OFF} by integrating the transient of charge that moved inward following the termination of the test pulse. The ratio $Q_{\text{ON}}/Q_{\text{OFF}}$ was 0.94 ± 0.13 ($n = 23$). Unless stated otherwise, $Q_{\max}$, the maximum amount of charge that could be moved, was taken as Q_{ON} for a +30 or +40 mV test pulse. In the standard protocol, test potentials were applied directly from the holding potential (-80 mV). The prepulse protocol (modified from ref. 19), consisted of a 1-s prepulse to -30 mV and a subsequent 20–40-ms repolarization to a pedestal potential (-50 mV), followed by depolarization to varying test potentials.

restored immobilization-resistant charge movements, with an average density ($Q_{\max}$) varying from 81% (pCAC6, pCsk3) to 195% (pCsk1) of that in normal myotubes (Fig. 2, Table 1). Presumably, this variation in density reflects differing levels of expression of the different cDNAs. The kinetics and voltage dependence of the restored immobilization-resistant charge were similar for the skeletal muscle, cardiac and five different chimaeric cDNAs. None of the restored charge movements manifested a Q_{γ} ('hump') component of the sort described for frog skeletal muscle⁹.

Taking immobilization-resistant charge movement as an indication of the level of expression of the cDNA-encoded DHP receptors, we used two criteria to compare the 'efficiency' of these different DHP receptors in producing L-type calcium conductance: first, the ratio of the maximum L-type conductance to the maximum quantity of immobilization-resistant charge (after correction for the assumed presence of $Q_{\text{dys-max}}$, the average, maximal, immobilization-resistant charge found in noninjected dysgenic myotubes); and second, the difference between the potential ($\bar{V}_G$) that activated the half-maximal L-type conductance and the potential ($\bar{V}_Q$) that moved half the immobilization-resistant charge. For pCAC6 the conductance-to-charge ratio was about 40% that of normal mouse myotubes (Table 1). The rightward (depolarized) shift of $\bar{V}_G$ relative to $\bar{V}_Q$ for pCAC6 (Fig. 3b) was much larger than for normal myotubes (Fig. 3a); from analysis of cells for which the estimated series resistance error was ≤ 10 mV, $\bar{V}_G - \bar{V}_Q$ was 40.8 ± 6.1 mV ($n = 7$) for pCAC6 and 18.3 ± 4.5 mV ($n = 6$) for normal myotubes. These differences cannot be ascribed to pCAC6 encoding the rabbit skeletal muscle DHP receptor, as results obtained from 11 rabbit myotubes were very similar to those from normal mouse myotubes. Because its expression is under the control of a constitutive promoter, the skeletal muscle DHP receptor produced in pCAC6-injected myotubes may differ from the DHP receptor in normal myotubes in its association with other subunits or in its post-translational modification¹⁰⁻¹².

Compared with pCAC6, expression of pCARD1 produced a larger conductance-to-charge ratio (Table 1), although this

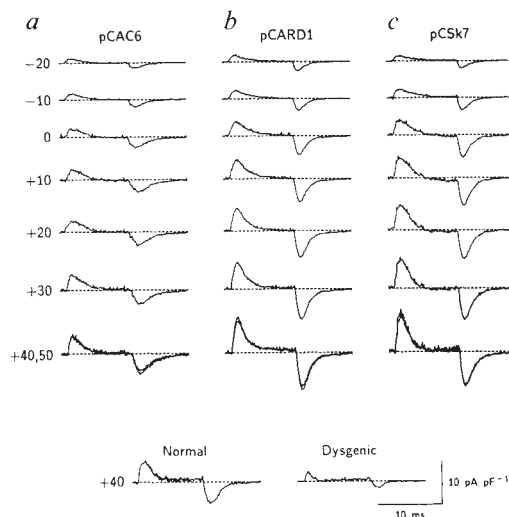


FIG. 2 Restoration of immobilization-resistant charge movement in dysgenic myotubes injected with pCAC6 (a), pCARD1 (b) or pCsk7 (c). Injection of pCsk1-4 resulted in charge movements similar to those illustrated here for pCAC6, pCARD1 and pCsk7. The inset illustrates representative immobilization-resistant charge movements measured from a normal myotube (left) and a non-injected dysgenic myotube (right). Immobilization-resistant charge movements were measured for the indicated test potentials using the prepulse protocol (Fig. 1A,2). a, Cell CA22, $C = 150$ pF. b, Cell CA44, $C = 425$ pF. c, Cell CA87, $C = 335$ pF. Inset: normal myotube BI26, $C = 310$ pF (left); dysgenic myotube BH82, $C = 210$ pF (right).

ratio was still smaller than that of normal myotubes. We have previously shown^{4,13}, for injected dysgenic myotubes, that the skeletal muscle DHP receptor controls the release of Ca^{2+} from the sarcoplasmic reticulum in a direct fashion, whereas the cardiac DHP receptor does not. But the ability or inability of a DHP receptor to mediate direct coupling does not seem to influence significantly its conductance-to-charge ratio (Table 1). The construct with the highest conductance-to-charge ratio (nearly twice that of normal myotubes) was pCsk3, which produce skeletal-type EC coupling. As exemplified by pCARD1 (Fig. 3c) and pCsk7 (Fig. 3d), the cDNAs encoding the entirely cardiac DHP receptor or chimaeric DHP receptors (in which the primary structure of the four repeat regions is predominantly cardiac) yielded a shift between $\bar{V}_G$ and $\bar{V}_Q$ that was much smaller than that for pCAC6; for the cardiac and chimaeric DHP receptors as a group, $\bar{V}_G - \bar{V}_Q$ was 12.3 ± 4.6 mV ($n = 9$, cells with series resistance error ≤ 10 mV).

By expressing cDNAs in dysgenic myotubes, we have shown here that the skeletal muscle DHP receptor is responsible for the bulk of intramembrane charge movement in normal skeletal muscle. This result, together with our previous demonstration that expression of the skeletal muscle DHP receptor cDNA restores EC coupling in dysgenic myotubes⁴, provides evidence that the skeletal muscle DHP receptor is the voltage sensor for EC coupling. Skeletal muscle, cardiac and chimaeric DHP receptor constructs all restore charge movements with very similar voltage-dependent behaviour, independent of the nature of the L-type current (slowly or rapidly activating) or EC coupling (skeletal- or cardiac-type) produced by them. This similarity in voltage dependence seems reasonable given that the S4 segments, which probably serve to sense voltage¹⁴⁻¹⁶, are highly conserved between the skeletal muscle² and cardiac⁷ DHP receptors.

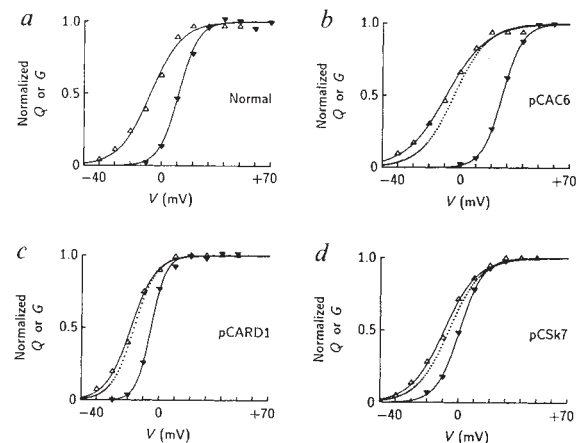


FIG. 3 Comparison of normalized, immobilization-resistant charge moved (Q , Δ) and normalized L-type calcium conductance activated (G , ∇) as a function of test potential (V) in a normal myotube (a) and dysgenic myotubes injected with pCAC6 (b), pCARD1 (c) or pCsk7 (d). Smooth curves represent the Boltzmann expression $1/[1 + \exp\{-(V - \bar{V})/k\}]$, where the parameters $\bar{V}_Q$, $\bar{V}_G$, k_Q and k_G were determined as described in Table 1 legend. Dashed curves represent fits of the Boltzmann expression (with parameters $\bar{V}'_Q$ and k'_Q) to the Q versus V relationships after correction of the Q values for the assumed presence of Q_{dys} , which has the magnitude and voltage dependence summarized in Table 1 for noninjected dysgenic myotubes. a, Cell BH74, $C = 240$ pF, estimated series resistance error (V_e) ≤ 3.6 mV, $Q_{\max} = 9.4$ nC μF^{-1} , $\bar{V}_Q = -7$ mV, $k_Q = 10.2$ mV, $\bar{V}_G = 11$ mV, $k_G = 6.5$ mV. b, Cell CA07, $C = 630$ pF, $V_e \leq 5.5$ mV, $Q_{\max} = 9$ nC μF^{-1} , $\bar{V}_Q = -9$ mV, $k_Q = 13.2$ mV, $\bar{V}'_Q = -4$ mV, $k'_Q = 11$ mV, $\bar{V}_G = 27$ mV, $k_G = 6.6$ mV. c, Cell CA37, $C = 450$ pF, $V_e \leq 13$ mV, $Q_{\max} = 11$ nC μF^{-1} , $\bar{V}_Q = -18$ mV, $k_Q = 7.8$ mV, $\bar{V}'_Q = -16$ mV, $k'_Q = 7.2$ mV, $\bar{V}_G = -5$ mV, $k_G = 4.6$ mV. d, Cell CA87, $C = 335$ pF, $V_e \leq 8$ mV, $Q_{\max} = 15.8$ nC μF^{-1} , $\bar{V}_Q = -9$ mV, $k_Q = 10.7$ mV, $\bar{V}'_Q = -6$ mV, $k'_Q = 10.1$ mV, $\bar{V}_G = 1$ mV, $k_G = 7.3$ mV.

Our results indicate that the conductance-to-charge ratio of the skeletal muscle DHP receptor is not very different from that of the cardiac DHP receptor, suggesting that the two types of DHP receptor do not appreciably differ in their ability to function as channels. Moreover, expression of chimaeric DHP receptors demonstrates that a DHP receptor can mediate skeletal-type EC coupling and also function as a channel with rapid activation⁸, large conductance-to-charge ratio and small shift between $\bar{V}_G$ and $\bar{V}_Q$. Thus, participation in direct, skeletal-type EC coupling does not necessarily cause a DHP receptor to lose its efficiency as a calcium channel. □

Received 30 March; accepted 5 June 1990.

- Rios, E. & Brum, G. *Nature* **325**, 717–720 (1987).
- Tanabe, T. *et al.* *Nature* **328**, 313–318 (1987).
- Beam, K. G., Knudson, C. M. & Powell, J. A. *Nature* **320**, 168–170 (1986).
- Tanabe, T., Beam, K. G., Powell, J. A. & Numa, S. *Nature* **336**, 134–139 (1988).
- Schneider, M. F. & Chandler, W. K. *Nature* **242**, 244–246 (1973).
- Knudson, C. M. *et al.* *J. biol. Chem.* **264**, 1345–1348 (1989).
- Mikami, A. *et al.* *Nature* **340**, 230–233 (1989).
- Tanabe, T., Beam, K. G., Adams, B. A., Nidome, T. & Numa, S. *Nature* **346**, 567–569 (1990).
- Adrian, R. H. & Peres, A. J. *Physiol.* **289**, 83–97 (1979).
- Catterall, W. A., Seagar, M. J. & Takahashi, M. *J. biol. Chem.* **263**, 3535–3538 (1988).
- Campbell, K. P., Leung, A. T. & Sharp, A. H. *Trends Neurosci.* **11**, 425–430 (1988).
- Perez-Reyes, E. *et al.* *Nature* **340**, 233–236 (1989).
- Tanabe, T., Mikami, A., Numa, S. & Beam, K. G. *Nature* **344**, 451–453 (1990).
- Noda, M. *et al.* *Nature* **312**, 121–127 (1984).
- Noda, M. *et al.* *Nature* **320**, 188–192 (1986).
- Stühmer, W. *et al.* *Nature* **339**, 597–603 (1989).
- Beam, K. G. & Knudson, C. M. *J. gen. Physiol.* **91**, 799–815 (1988).
- Hamill, O. P., Marty, A., Neher, E., Sakmann, B. & Sigworth, F. J. *Pflügers Arch. ges. Physiol.* **391**, 85–100 (1981).
- Bean, B. P. & Rios, E. *J. gen. Physiol.* **94**, 65–93 (1989).

ACKNOWLEDGEMENTS. We thank L. VanZuiden and A. Beam for providing cultured myotubes. This research was supported in part by the Ministry of Education, Science and Culture of Japan, the Mitsubishi Foundation, the Japanese Foundation of Metabolism and Diseases, the NIH, and the Muscular Dystrophy Association. B.A.A. was supported by an NIH postdoctoral fellowship.

Specific recognition of staphylococcal enterotoxin A by human T cells bearing receptors with the V γ 9 region

Chantal J. J. Rust, Frank Verreck, Henk Vietor & Frits Koning*

Department of Immunohematology and Bloodbank, PO Box 9607, University Hospital, 2300 RC Leiden, The Netherlands

T CELLS bearing the $\alpha\beta$ receptor can specifically react with target cells coated with staphylococcal enterotoxin and expressing major histocompatibility complex class II molecules; these responses depend on which variable region (V) of the receptor's β -subunit is used^{1–7}. We have now examined whether a similar situation exists for human T cells bearing the $\gamma\delta$ receptor. We found that reactivity to staphylococcal enterotoxin A is strictly dependent on the presence of the V γ 9 variable region in the $\gamma\delta$ T-cell receptor (TCR). These cytotoxic responses required the expression of HLA class II molecules by the target cell and could be inhibited by anti- $\gamma\delta$ TCR and by anti-HLA-class-II monoclonal antibodies. In contrast to $\alpha\beta$ TCR⁺ cell clones, no proliferative response of V γ 9⁺ T-cell clones towards stimulator cells coated with enterotoxin A was observed *in vitro*. These results indicate that the $\gamma\delta$ TCR repertoire might be influenced by enterotoxin A produced during staphylococcal infections *in vivo*. This could provide a molecular basis for the observation that V γ 9⁺ T cells form the large majority of peripheral $\gamma\delta$ TCR⁺ cells but only a small proportion of thymic $\gamma\delta$ TCR⁺ cells.

To test whether $\gamma\delta$ TCR⁺ cells can react to target cells coated with enterotoxin we have used a panel of $\gamma\delta$ TCR⁺ T-cell clones. These clones have previously been characterized by their usage of the V γ 9, V δ 1 and V δ 3 V-regions, using monoclonal antibodies specific for those V-regions^{8,9}. Six $\gamma\delta$ TCR⁺ clones and one $\alpha\beta$ TCR⁺ clone were tested in a cell-mediated lympholysis (CML) assay against a B-lymphoblastoid cell line (BLCL) transformed by Epstein-Barr virus, which had been preincubated with enterotoxin A (0–10^{−6} g ml^{−1}). Specific, dose-dependent killing of enterotoxin-coated target cells was observed at toxin concentrations greater than 10^{−8} g ml^{−1} with some of the T cell clones but not with others (Fig. 1a). The specificity of the reactivity against enterotoxin-coated target cells is further stressed by the observation that lowering the effector-to-target ratio decreases specific lysis (Fig. 1b). In this small panel of six $\gamma\delta$ TCR⁺ clones, specific lysis was observed only if the clones expressed the V γ 9 V-region (clones HV.JH10, AH21, LH22 and LH3), and so we subsequently tested a larger panel of $\gamma\delta$ TCR expressing clones derived from peripheral blood and thymus for reactivity against targets preincubated with enterotoxin (Table 1). Again, specific killing was observed only if the T cell clones expressed the V γ 9 V-region, either in combination with

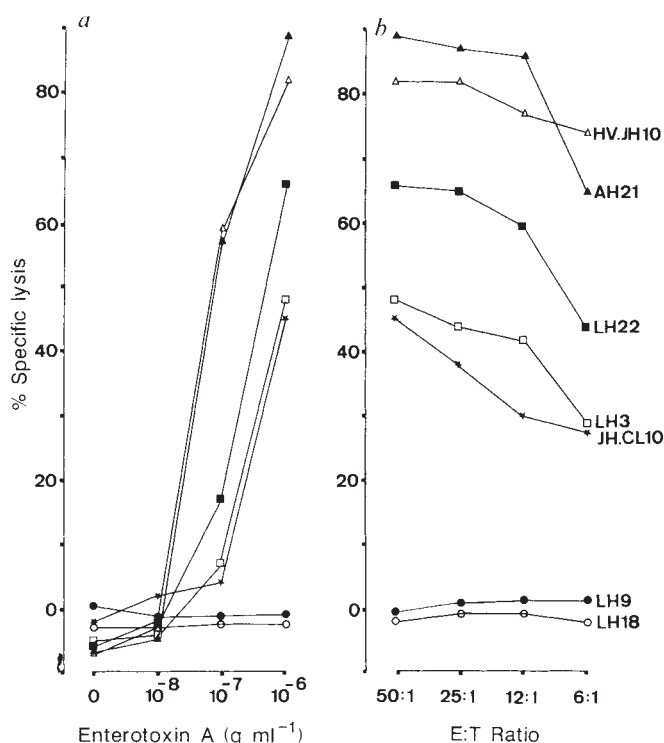


FIG. 1 Cytotoxic activity of $\gamma\delta$ TCR⁺ T cell clones against target cells coated with staphylococcal enterotoxin A. a, Dose-dependent effect of enterotoxin on cytotoxic activity of $\gamma\delta$ TCR⁺ and $\alpha\beta$ TCR⁺ T cell clones. Isolation of T cell clones has been described previously^{8,9}. Effector cells used were (see b): HV.JH10 (V γ 9⁺V δ 1⁺V δ 3⁺), AH21 (V γ 9⁺V δ 1⁺V δ 3⁺), LH22 (V γ 9⁺V δ 1⁺V δ 3⁺), LH3 (V γ 9⁺V δ 1⁺V δ 3⁺), LH9 (V γ 9⁺V δ 1⁺V δ 3⁺), LH18 (V γ 9⁺V δ 1⁺V δ 3⁺), JH.CL10 ($\alpha\beta$). Target cells (BLCL Plicht, DR2 DR3) were preincubated with enterotoxin 0–10^{−6} g ml^{−1} (Toxin Technology Inc., WI) for 1 h at 37 °C. After incubation the cells were spun down and washed once. Effector cells were incubated in 96-well round bottom microtitre plates with 5 × 10³ [⁵¹Cr]sodium chromate-labelled target cells at an effector-to-target cell ratio of 50:1 in a standard CML assay as described previously³². Assay time was 4 h. Per cent specific lysis was calculated as

$$100 \times \frac{\text{test c.p.m.} - \text{spontaneous release}}{\text{maximum c.p.m.} - \text{spontaneous release}}$$

b, Specific lysis of enterotoxin-coated target cells at different effector-to-target cell ratios. Serial dilutions of effector cells were incubated with target cells coated with enterotoxin A (10^{−6} g ml^{−1}). Cells used and method as in a.

* To whom correspondence should be addressed.

TABLE 1 Cytotoxic activity of a panel of $\gamma\delta$ TCR⁺ T cell clones and an $\alpha\beta$ TCR⁺ T cell clone against enterotoxin-coated target cells and the natural killer cell sensitive target cell line K562

Clone*	V γ 9/V δ 1/V δ 3 [†]	S-S [‡]	Experiment 1			Experiment 2		
			BLCL		K562	BLCL		K562
			—	Enterotoxin		—	Enterotoxin	
JH1	+++	+	-6 [§]	33	-6	-5	54	15
HV.JH10	+++	ND	-3	76	60	-5	92	53
LH1	+++	ND	6	76	70		ND	
LH32	+++	ND	3	83	7	2	90	ND
MH5	+++	ND	-1	26	10	2	75	56
RdVw1.CD4	+++	+	-4	51	0	-5	36	12
PH3	+++	ND	-2	67	80		ND	
LH3	++-	+	-7	27	-5	-9	72	17
LH8	++-	+	-1	60	33	-9	49	17
LH14	++-	+	-6	30	-2	-9	32	21
LH22	++-	+	-5	32	-2	-8	47	20
AH21	++-	ND	-4	76	32	-7	89	45
PH20	++-	ND	-1	66	-5	6	85	ND
PH27	++-	ND	8	90	70		ND	
ThyC15	++-	-	7	82	65	0	60	33
ThyC110	++-	+	6	93	42	1	91	28
PH22	++-	ND	-3	71	78		ND	
ThyC16	++-	-	7	94	55		ND	
JHC12	-+-	-	0	7	80		ND	
PHC130	-+-	-	0	3	34		ND	
LH9	-+-	-	-5	-2	63	-4	-7	69
LH16	-+-	-	-4	-1	46	-4	-3	56
LH18	-+-	-	-1	1	68	0	-2	75
AH19	-+-	ND	-2	2	56	-4	-3	33
JH13	-+-	-	-5	-1	52	-2	1	62
AH26a	-+-	ND	3	5	66		ND	
ThyC13	---	+	-4	3	70		ND	
JHC18	---	-	2	7	90		ND	
JHC110	$\alpha\beta$	+	-4	20	-3		ND	

BLCL (Plicht, DR2 DR3) was preincubated with 10^{-6} g ml⁻¹ enterotoxin A. The erythroblastoid cell line K562 was used as a control for the natural killer activity of the T cell clones. The expression of the V γ 9, V δ 1 and V δ 3 V-regions has been determined previously^{8,9} and is indicated in the table as well as the presence or absence of a disulphide bridge between the TCR γ and δ chain. Results are shown for the E:T ratio of 50:1 only. CML assay was as described in the legend to Fig. 1a.

* With the exception of LH1 and RdVw1.CD4, all clones lack the expression of CD4.

[†] Usage of the V regions determined by the use of the monoclonal antibodies T γ A (V γ 9)¹⁴, δ TC1 (V δ 1)¹² and BB3 (V δ 3)³¹.

[‡] Presence of a disulphide bridge between the γ and δ chain of the T-cell receptor has been determined by SDS-PAGE.

[§] Per cent specific lysis. ND, not done.

V δ 1, V δ 3 or with unknown V δ V-regions (Table 1). None of the V γ 9⁺ $\gamma\delta$ TCR⁺ clones lysed enterotoxin-coated target cells; however, such clones usually did lyse the cell line K562 which is sensitive to natural killer cells (Table 1). The failure to kill enterotoxin-coated targets is therefore not due to an intrinsic inability to lyse appropriate targets but rather reflects a failure specifically to interact with enterotoxin A.

Previous studies¹⁰⁻¹⁵ have shown that V γ 9⁺ $\gamma\delta$ TCRs are usually disulphide-linked (C γ 1 encoded¹⁶⁻¹⁸), whereas most V γ 9⁺ V δ 1⁺ $\gamma\delta$ TCRs are not disulphide linked (C γ 2 encoded¹⁶⁻¹⁸; see also Table 1). To exclude the possibility that specific lysis of enterotoxin-coated target cells could be accounted for by the usage of C γ 1 in V γ 9⁺ $\gamma\delta$ TCR complexes, we have used two thymus-derived clones (ThyC15 and ThyC110) that both express V γ 9 but differ in the usage of the C γ region, as indicated by the presence or absence of a disulphide bridge between the TCR γ and δ chains (Table 1). As both clones lyse enterotoxin-coated target cells, this reactivity is not dependent on the use of either the C γ 1 or C γ 2 gene segments.

To test whether the $\gamma\delta$ TCR⁺ clones would lyse BLCL that had been preincubated with enterotoxins other than A, a number of clones were tested against a BLCL preincubated with staphylococcal enterotoxins A, B, C1 or the enterotoxin-related protein, TSST¹⁹. None of the clones, with the exception of LH8 (V γ 9⁺ V δ 1⁺), lysed BLCL cells preincubated with B, C1 or TSST, whereas targets coated with enterotoxin A were specifically lysed by the T cell clones expressing V γ 9 (Table 2). Thus, in this panel of clones, the reactivity of $\gamma\delta$ TCR-expressing T cell clones

against enterotoxin seems to be almost exclusively towards enterotoxin A. The reactivity towards enterotoxin-coated targets could be inhibited by anti-HLA-class II and anti- $\gamma\delta$ TCR receptor monoclonal antibodies but not by those against HLA-class I and $\alpha\beta$ TCR (results not shown), indicating that both the $\gamma\delta$ TCR and HLA-class II are necessary for recognition of enterotoxin. This dependence on HLA-class II expression is further indicated by the inability of the $\gamma\delta$ TCR⁺ clones to lyse HLA-class II negative target cells that had been preincubated with enterotoxin A (results not shown). The HLA class II phenotype of the target cells appears, however, to be irrelevant as V γ 9⁺ T cell clones lyse target cells matched and mismatched for HLA class II and which have been preincubated with enterotoxin A (results not shown).

Whereas human $\alpha\beta$ TCR⁺ cells can proliferate in response to and also lyse enterotoxin-coated target cells⁴⁻⁶, we have so far been unable to demonstrate proliferation of cells bearing V γ 9⁺ $\gamma\delta$ TCR in response to enterotoxin A (Table 3). In the same experiment, the $\alpha\beta$ TCR⁺ clone JH.C110 did proliferate in response to stimulator cells coated with enterotoxin A (Table 3). Thus, although both $\alpha\beta$ TCR⁺ and $\gamma\delta$ TCR⁺ cells can respond to staphylococcal enterotoxins, the response of the former may be both cytotoxic and proliferative whereas the latter seem chiefly to mount a cytotoxic response.

As the C δ gene segment is located within the TCR α -locus and C α and C δ are most probably the result of a gene duplication²⁰, it seems likely that the α and δ chains are each other's homologue. Similarly, the γ chain is probably the homologue

TABLE 2 Cytotoxic activity of $\gamma\delta$ TCR⁺ T cell clones against target cells coated with enterotoxin A, B, C1 and TSST

Clone	V γ 9/V δ 1/V δ 3	% Lysis				
		—	SEA	SEB	SEC1	TSST
JH1	++	-6	33	-6	5	-4
HVJH10	++	-3	76	-2	-3	-5
RdVw1.CD4	++	-4	51	1	-1	-4
MH5	++	-1	26	-2	1	-7
LH3	++	-7	27	-3	-4	-5
LH8	++	-1	60	18	26	6
LH14	++	-6	30	-3	1	-3
LH22	++	-5	32	1	3	-3
AH21	++	-4	76	3	4	-3
PH22	++	-3	71	0	9	-1
LH9	++	-5	-2	-5	-1	-3
LH16	++	-4	-1	2	1	-6
LH18	++	-1	1	0	1	-3
AH19	++	-2	2	-1	8	-3
JH13	++	-5	-1	-2	2	-4
JH.C110	$\alpha\beta$	-4	20	-1	-4	-7

BLCL (Plicht, DR2, DR3) was preincubated with medium (—) or with enterotoxins (10⁻⁶ g ml⁻¹), B (10⁻⁶ g ml⁻¹), C1 (10⁻⁶ g ml⁻¹) or TSST (10⁻⁶ g ml⁻¹). CML assay as in legend to Fig. 1a.

TABLE 3 Proliferative response of $\gamma\delta$ TCR⁺ and $\alpha\beta$ TCR⁺ T cell clones against stimulator cells coated with enterotoxin A

T cell clone	Enterotoxin concentration					IL-2
	—	10 ⁻⁹	10 ⁻⁸	10 ⁻⁷	10 ⁻⁶	
MH5 ($\gamma\delta$)	437	480	563	592	460	15,093
JH1 ($\gamma\delta$)	515	575	957	971	815	62,383
LH22 ($\gamma\delta$)	870	683	638	788	433	43,867
LH1 ($\gamma\delta$)	635	627	588	607	545	4,735
JH.C110 ($\alpha\beta$)	597	653	45,290	55,627	52,087	30,830
—*	605	600	648	657	528	553

The $\gamma\delta$ TCR⁺ T cell clones MH5 (V γ 9⁺V δ 1⁺V δ 3⁺), JH1 (V γ 9⁺V δ 1⁺V δ 3⁺), LH22 (V γ 9⁺V δ 1⁺V δ 3⁺), LH1 (V γ 9⁺V δ 1⁺V δ 3⁺) and the $\alpha\beta$ TCR⁺ T cell clone JH.C110 were tested for proliferation in response to enterotoxin A-coated (0–10⁻⁶ g ml⁻¹) BLCL (Plicht, DR2, DR3) or to interleukin-2 (IL-2) (30 U ml⁻¹). Proliferative responses were measured in triplicates of 200 μ l containing 10⁴ cloned T cells as responders and 5 $\times$ 10⁴ irradiated BLCL (4,500 R) as antigen-presenting cells in flat bottomed 96-well microtitre plates. After 72 h the cultures were pulsed with [³H]thymidine (NEI: 1.0 μ Ci per well) for 16 h. The results are expressed as mean c.p.m. of triplicates.

* BLCL alone.

of the β chain in $\gamma\delta$ TCR and $\alpha\beta$ TCRs respectively. As the response of $\alpha\beta$ TCR⁺ cells to enterotoxin is controlled by the expression of V β gene segments, our results indicate that V β and V γ act as functional homologues, determining the reactivity against enterotoxins.

Considering that only cells bearing V γ 9⁺ $\gamma\delta$ TCR respond to enterotoxin A, it is of interest that V γ 9⁺ cells constitute the large majority of $\gamma\delta$ TCR⁺ cells in the periphery but only a minor fraction of this T-cell subset in the thymus^{8,10–12,14,21,22}. Our results argue against a large deletion of V γ 9-bearing cells on the basis of selection by self 'superantigens' as has been described for Mls- and I-E-reactive T cells^{23–26}. Rather, an extrathymic expansion of V γ 9-bearing cells induced by staphylococcal enterotoxins present during bacterial infections might account for the predominant expression of V γ 9 in the periphery and for the relative lack of V γ 9 expression in the thymus. The observation that $\gamma\delta$ TCR⁺ cells do not proliferate *in vitro* in response to enterotoxin A may not be valid in the *in vivo* situation as the cells expressing $\gamma\delta$ TCR might benefit from growth factors produced by $\alpha\beta$ TCR⁺ cells responsive to enterotoxin, as they do *in vitro* (Table 3). It may be relevant that cells expressing $\gamma\delta$ TCR are often found within epithelia in the skin^{27,28}, gut²⁹ and lung³⁰, where minimal tissue damage may result in exposure to staphylococcal enterotoxins.

Our results show that $\gamma\delta$ TCR⁺ cells, like $\alpha\beta$ TCR⁺ cells, can specifically recognize staphylococcal enterotoxin A. This recognition is dictated by the expression of the V γ 9 gene segment

and requires HLA class II expression by the target cell. Such specific responses may have a profound influence on the selection of the $\gamma\delta$ TCR repertoire. □

Received 21 February; accepted 24 May 1990.

- Janeway, C. A. & Katz, M. E. *J. Immunol.* **134**, 2057–2063 (1985).
- Lynch, D. H., Cole, B. C., Bluestone, J. A. & Hodes, R. J. *Eur. J. Immunol.* **16**, 747–751 (1986).
- Janeway, C. A. *et al. Immunol. Rev.* **107**, 61–88 (1989).
- Fleischer, B. & Schrezenmeier, H. *J. exp. Med.* **167**, 1697–1708 (1988).
- Kappler, J. W. *et al. Science* **244**, 811–813 (1989).
- Fleischer, B., Schrezenmeier, H. & Conradt, P. *Cell Immunol.* **120**, 92–101 (1989).
- White, J. *et al. Cell* **56**, 27–35 (1989).
- Vietor, H. & Koning, F. *Immunogenetics* (in the press).
- Koning, F., Knot, M., Wassenaar, F. & v.d. Elsen, P. *Eur. J. Immunol.* **19**, 2099–2105 (1989).
- Bottino, C. *et al. J. exp. Med.* **168**, 491–505 (1988).
- Borst, J. *et al. Eur. J. Immunol.* **19**, 1559–1568 (1989).
- Lanier, L. *et al. Eur. J. Immunol.* **18**, 1985–1992 (1988).
- Jitsukawa, S. *et al. J. exp. Med.* **166**, 1192–1197 (1987).
- Triebel, F. *et al. J. exp. Med.* **167**, 694–699 (1988).
- Faure, F., Jitsukawa, S., Triebel, F. & Hercend, T. *J. Immunol.* **140**, 1372–1379 (1988).
- Littman, D. R. *et al. Nature* **326**, 85–88 (1987).
- Krangel, M. S., Band, H., Hata, S., McLean, J. & Brenner, M. B. *Science* **237**, 64–67 (1987).
- Pellico, P. G., Subar, M., Weiss, A., Dalla-Favera, R. & Littman, D. R. *Science* **237**, 1051–1055 (1987).
- Bergdoll, M. S. in *Staphylococci and Staphylococcal Infections* (eds Easmon, S. C. F. & Adlam, C.) 559–598 (Academic, London, 1983).
- Chien, Y., Iwashima, M., Kaplan, K. B., Eliot, J. F. & Davis, M. M. *Nature* **327**, 677–682 (1987).
- Faure, F., Jitsukawa, S., Triebel, F. & Hercend, T. *J. Immunol.* **141**, 3357–3360 (1988).
- Mingari, M. C. *et al. Eur. J. Immunol.* **18**, 1831–1834 (1988).
- Kappler, J. W., Roehm, N. & Marrack, P. *Cell* **49**, 273–280 (1987).
- Kappler, J. W., Staerz, U. D., White, J. & Marrack, P. *Nature* **332**, 35–40 (1988).
- MacDonald, H. R. *et al. Nature* **332**, 40–45 (1988).
- Marrack, P. & Kappler, J. W. *Nature* **332**, 840–843 (1988).
- Koning, F. *et al. Science* **236**, 834–837 (1987).
- Kuziel, W. A. *et al. Nature* **328**, 263–266 (1987).
- Goodman, T. & Lefrançois, L. *Nature* **333**, 855–858 (1988).
- Augustin, A., Kubo, R. T. & Sim, G.-K. *Nature* **340**, 239–241 (1989).
- Triebel, F. *et al. Eur. J. Immunol.* **18**, 2021–2027 (1988).
- Goulmy, E. in *HLA Typing: Methodology and Clinical Aspects*, 105–122 (CRC, New York, 1982).

ACKNOWLEDGEMENTS. We thank Drs J. Borst, T. Hercend and L. Moretto for monoclonal antibodies, Drs R. P. P. de Vries, T. H. M. Ottenhoff and J. J. van Rood for critically reading the manuscript and W. C. A. van Schooten for helpful suggestions.

Quantitation of antigen-presenting cell MHC class II/peptide complexes necessary for T-cell stimulation

Clifford V. Harding & Emil R. Unanue

Department of Pathology, Washington University School of Medicine, St Louis, Missouri 63110, USA

THE number of specific complexes formed between peptide and the class II major histocompatibility complex (MHC) molecules expressed by an antigen-presenting cell (APC) after exposure to protein antigens is unknown, as is the number that activates T cells. Presentation of foreign peptides by APC takes place when many class II molecules may be occupied by autologous peptides^{1–3}. We have now estimated the number of specific peptide/class II complexes per APC by quantitative immunoprecipitation of I-A^k after pulsing the APC with stimulatory levels of a radioactive immunogenic peptide derived from hen egg-white lysozyme protein. T cells were activated by APC that expressed as few as 210–340 specific peptide/class II complexes (0.1% of the I-A^k molecules). These figures were confirmed using anti-CD3 antibody bound to latex beads as an alternative activating ligand. This low number explains the simultaneous presentation of multiple foreign antigens, even in the face of peptide competition.

For this study, we selected a T-cell hybridoma, 3A9, because its response is minimally dependent on the presence of adhesion or co-stimulator molecules. (3A9 cells¹, and some other T-cell hybridomas^{4,5}, are activated by specific peptide/class II complexes expressed directly on a lipid monolayer.) We first established the amounts of the hen egg-white lysozyme (HEL) peptide (peptide Y-E-52-61) that stimulate 3A9 T cells. Pulsing viable

TABLE 1 Parameters in the binding of peptides by class II MHC molecules

A	K_d : HEL(Y-E-52-61)+I-A ^k	10^{-7} M
B	Total I-A ^k per TA3 cell (I-A ^k _{tot})	2×10^5 molecules
C	Minimum stimulatory peptide concentration	10^{-7} M
D	Observed complexes per cell (10^{-7} M peptide)	210 (0.1% of I-A ^k _{tot})
E	Expected complexes per cell (without competition, 10^{-7} M peptide)	10^5 (50% of I-A ^k _{tot})
F	Calculated competing peptide concentration	10^{-4} M
G	Potential total peptide concentration	3×10^{-3} M

These calculations assume equilibrium conditions; careful dose-response studies indicate that APC present maximally to T cells after a peptide pulse of 4 h (data not shown). Turnover of peptide/I-A^k complexes is rapid in viable TA3 cells ($t_{1/2} = 15-50$ min)¹⁵. K_d , dissociation constant. A, see ref. 16. B, see ref. 6. C, D, The threshold number of HEL(Y-E-52-61)/I-A^k complexes per APC for T-cell (3A9) stimulation is 210. This level of complex is generated in viable TA3 cells after incubation with 100 nM peptide (Fig. 2). E, If competition was absent

$$Y_p = \frac{[P]}{[P] + K_d}$$

where Y_p is the fractional I-A^k occupancy by peptide P (ref. 17). F, Calculations for the amount of competitor (C) that would account for the results of D (ref. 17):

$$Y_p = \frac{P/I-A^k}{I-A^k_{tot}} = \frac{[P]}{[P] + K_{dp}(1 + [C]/K_{dc})}$$

Assuming $K_{dc} \approx K_{dp}$:

$$\frac{P/I-A^k}{I-A^k_{tot}} = \frac{[P]}{[P] + K_{dp} + [C]}$$

Substituting values from parts A-D above:

$$\frac{2 \times 10^2 \text{ per cell}}{2 \times 10^5 \text{ per cell}} = \frac{10^{-7} \text{ M}}{10^{-7} \text{ M} + 10^{-7} \text{ M} + [C]}$$

and $C \approx 10^{-4}$ M. G. The total potential concentration of peptides derived from extracellular protein (see text).

TA3 B-hybridoma cells (which express $\sim 2 \times 10^5$ molecules each of I-A^k and I-A^d)⁶ with 100 nM peptide for 4 h generated a small 3A9 response; pulsing with 316 nM peptide produced a vigorous response (Fig. 1). Next we pulsed the viable TA3 cells with similar amounts of radioactive peptide (radioactive and unlabelled peptide bind equally to I-A^k) and directly measured the number of peptide/I-A^k complexes by quantitative immunoprecipitation of I-A^k (Fig. 2). Nonspecific binding was assessed by the precipitation of I-A^d, which does not bind this peptide⁷, yielding only 2% of the amount of peptide bound to I-A^k. Pulsing with the two stimulatory peptide concentrations (that is, 100 and 316 nM) resulted in 210 and 340 molecules of I-A^k-bound peptide per TA3 cell, respectively (Fig. 2). These figures are averages for the cell populations, but our data indicate that variation in the expression of class II molecules or T-cell receptors is relatively minor in these cells. Additional studies of ours indicate that other T-cell hybridomas and T-cell clones are similarly sensitive to peptide/class II complexes.

We compared these results with an estimation of the minimum number of anti-CD3 antibodies bound to a latex bead that would trigger the T-cell hybridoma. A single latex bead containing 140 bivalent antibody molecules activated 3A9 cells (Fig. 3). Thus, the threshold for T-cell activation was ligation of less than 300 receptors, a figure compatible with the results of Figs 1 and 2, and with other reports⁸⁻¹².

The number of peptide/class II complexes that triggers the T cells represents only 0.1-0.2% of the I-A^k molecules expressed

by the TA3 cells (Table 1). Most of the I-A^k molecules were not occupied by the HEL peptide, even when pulsing at concentrations close to the affinity constant. The most likely explanation is that the I-A^k molecules were already occupied, or being competed for, by other peptides. Reverse phase HPLC of cell-associated peptides showed that most of the ¹²⁵I-Y-E-52-61/peptide remained intact during the incubation at 37 °C (data not shown), excluding the possibility that rapid peptide degradation¹³ resulted in a lower effective peptide concentration.

In these experiments viable TA3 cells were cultured in medium containing serum. Most serum proteins do not compete for binding to MHC molecules unless they are catabolized to peptides¹⁴. Peptide catabolites of these proteins produced intracellularly may already occupy the I-A^k molecules and may also compete for binding of the HEL peptide to I-A^k in an intracellular compartment. Competing peptides with similar affinity must be present at a concentration of 100 μM to fully account for the low level of binding (Table 1). Indeed, TA3 cells incubated with peptide in the absence of serum showed greatly increased numbers of complexes (Fig. 2b). The magnitude of this increase implies an 86% reduction in competing peptide concentration (to 1.4×10^{-5} M, calculated as in Table 1), indicating that most of the competing peptides are produced from exogenous proteins (for example, serum proteins).

Assuming complete conversion of serum proteins to peptides of relative molecular mass 2,000 (M_r 2K), medium with 10% serum could generate a peptide concentration of 3 mM, enough to produce the level of competition calculated above, even if only a small fraction of the peptides bind class II molecules. Even in the absence of serum, the number of specific complexes generated represents a fraction of the total number of I-A^k molecules available. A residual level of peptide competition may be due to degradation of endogenous proteins and the uptake of secreted or other remaining extracellular proteins. A low degree of peptide binding to I-A^k molecules also takes place on fixed cells. Only about 2% of surface class II molecules on fixed cells are available to bind peptide (calculated as in Table 1), but peptide competition is much lower at the cell surface.

We conclude that expression of only 200-300 complexes is sufficient to stimulate a T cell. The actual number of T-cell receptors ligated by peptide/class II complexes may be less, as not all of the 200-300 complexes on an APC may bind a T-cell

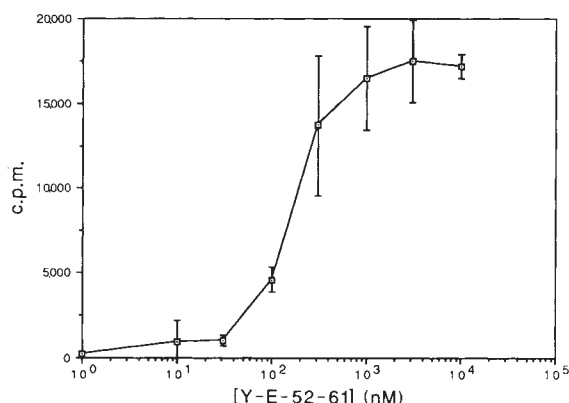


FIG. 1. Presentation of the HEL peptide Y-E-52-61 to 3A9 T-hybridoma cells. The APC were the TA3 line, a B-cell hybridoma that expresses I-A^k and I-A^d. The HEL peptide is presented by I-A^k. Viable TA3 cells were incubated with HEL(Y-E-52-61) for 4 h at 37 °C, then fixed and incubated (2×10^5 per well) with 3A9 cells (10^5 per well). The resultant interleukin-2 (IL-2) secretion was measured by a bioassay using the IL-2-dependent CTL cell line⁵.

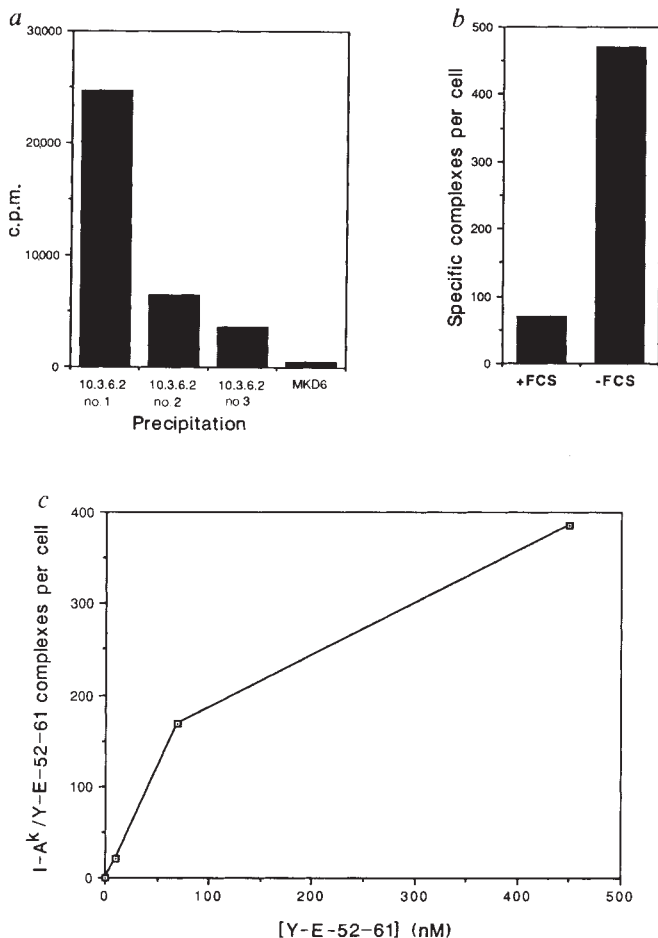
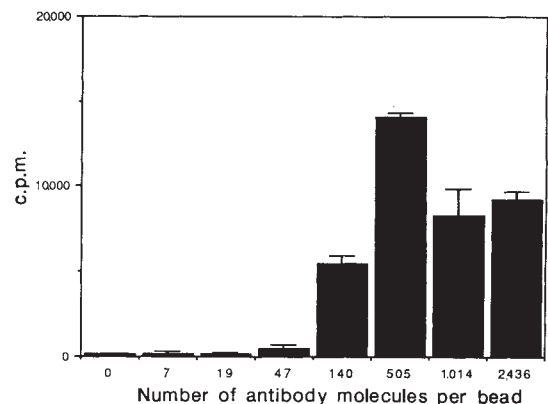


FIG. 3 Stimulation of 3A9 cells with anti-CD3 coupled to beads. Various amounts of ^{125}I -labelled 145-2C11 (anti-CD3 monoclonal antibody) (ref. 18) (10^6 c.p.m. μg^{-1}) were conjugated to 6- μm beads (Polysciences) by incubation at 37°C for 4 h, followed by washing. In a first experiment (data not shown) the beads were incubated at varying titres with 3A9 T-cells (10^5 per well) for 18 h, and the resulting IL-2 production assayed. The IL-2 bioassay was not sensitive to the response of the number of T cells stimulated by 100 or fewer beads per well. The number of beads bound per cell was determined microscopically. Using 300 beads per well, usually only a single bead was associated with a given T cell at one time and beads with 140 bivalent antibodies were stimulatory (above).

receptor—indeed, a minority of the complexes are probably intracellular⁶. The remarkable sensitivity of the T-cell response implies that an immunogenic peptide need only occupy a small fraction of the available class II molecules. Thus, an APC can

FIG. 2 Immunoprecipitation of ^{125}I -labelled Y-E-52-61/I-A^{*} complexes from TA3 cells. **a**, TA3 cells were incubated with ^{125}I -labelled Y-E-52-61 for 4 h at 37°C . The iodinated peptide, purified by reverse phase HPLC, had a specific activity of 6×10^{18} c.p.m. mol^{-1} ; Y-E-52-61 and ^{125}I -labelled Y-E-52-61 bind equally to I-A^{*} (ref. 16). The cells were washed and solubilized in 1% Triton X-100 in PBS buffer with leupeptin ($10 \mu\text{g ml}^{-1}$), phenylmethylsulphonylfluoride ($0.2 \mu\text{M}$) and iodoacetamide (10 mM). I-A^{*} was immunoprecipitated by several sequential incubations with anti-I-A^{*} antibody (10.3.6.2) and protein A/Sepharose (CL-4B). The first antibody was added either before or after cell solubilization (the former resulted in a higher yield in the first immunoprecipitation). Five to six sequential precipitations were necessary to precipitate all of the I-A^{*} (not shown); the results of three rounds are shown (no. 1, no. 2 and no. 3), which precipitated 81% of the soluble I-A^{*} (58, 15 and 8%, respectively) and constituted the standard protocol. The efficiency of I-A^{*} solubilization was 87%, as revealed by solubilization of TA3 cells after labelling of I-A^{*} with ^{125}I -labelled 10.3.6.2; thus, the cumulative efficiency of I-A^{*} immunoprecipitation from cells was 70%. To demonstrate further that this procedure results in essentially complete I-A^{*} precipitation, affinity-purified I-A^{*} was complexed with radioactive peptide^{15,16}; most of these complexes were immunoprecipitated in 2–3 rounds by this protocol. MKD6, the immunoprecipitation of I-A^{*} (also expressed by TA3 cells). Only the first immunoprecipitation is indicated here, with very low levels of associated radioactive peptide. **b**, Level of ^{125}I -labelled Y-E-52-61/I-A^{*} complexes measured as above, except that TA3 cells were incubated in the presence and absence of 10% FCS for 2 h at 37°C and $0.05 \mu\text{M}$ peptide was then added for 2 h at 37°C , followed by immunoprecipitation with 10.3.6.2. **c**, Specific curve: number of Y-E-52-61/I-A^{*} complexes per cell (corrected for 70% yield of immunoprecipitation procedure) after pulsing with the peptide as in **a**.



simultaneously present multiple foreign peptides even in the face of self-peptide competition. T cells have evolved to respond to very small amounts of peptide antigens, as a limited number of MHC molecules are bound by a given peptide. □

Received 12 March; accepted 1 June 1990.

1. Babbitt, B. P., Matsueda, G., Haber, E., Unanue, E. R. & Allen, P. M. *Proc. natn. Acad. Sci. U.S.A.* **83**, 4509–4513 (1986).
2. Lorenz, R. G. & Allen, P. M. *Proc. natn. Acad. Sci. U.S.A.* **85**, 5220–5224 (1988).
3. Buus, S., Sette, A., Colon, S. M. & Grey, H. M. *Science* **242**, 1045–1047 (1988).
4. Fox, B. S., Quill, H., Carlson, L. & Schwartz, R. H. *J. Immun.* **138**, 3367–3373 (1987).
5. Watts, T. J. *Immun.* **141**, 3708–3716 (1988).
6. Harding, C. V. & Unanue, E. R. *J. Immun.* **142**, 12–19 (1989).
7. Babbitt, B. P., Allen, P. M., Matsueda, G., Haber, E. & Unanue, E. R. *Nature* **317**, 359–361 (1985).
8. Braciale, T. J., Morrison, L. A., Herkel, T. J. & Braciale, V. L. in *Processing and Presentation of Antigens* (eds Pernis, B., Silverstein, S. C. & Vogel, H. J.) 69–79 (Academic, San Diego, 1988).
9. Romani, N. *et al. J. exp. Med.* **169**, 1153–1168 (1989).

10. Kanagawa, O. & Ahlert, C. *Int. Immun.* **1**, 565–569 (1989).
11. Lanzavecchia, A. *Immunol. Rev.* **99**, 39–51 (1987).
12. Demotz, S., Grey, H. M., Apella, E. & Sette, A. *Nature* **342**, 682–684 (1989).
13. Fox, B. S., Carbone, F. R., Germain, R. N., Paterson, Y. & Schwartz, R. H. *Nature* **331**, 538–540 (1988).
14. Lorenz, R. G., Blum, J. S. & Allen, P. M. *J. Immun.* **144**, 1600–1606 (1990).
15. Harding, C. V., Roof, R. W. & Unanue, E. R. *Proc. natn. Acad. Sci. U.S.A.* **86**, 4230–4234 (1989).
16. Roof, R. W., Luescher, I. F. & Unanue, E. R. *Proc. natn. Acad. Sci. U.S.A.* **87**, 1735–1739 (1990).
17. Limbird, L. E. *Cell Surface Receptors: A Short Course on Theory and Methods* (Martinus Nijhoff, Boston, 1986).
18. Leo, O., Foo, M., Sacks, D. H., Samelson, L. E. & Bluestone, J. A. *Proc. natn. Acad. Sci. U.S.A.* **84**, 1374–1378 (1987).

A morphogenetic gradient of *hunchback* protein organizes the expression of the gap genes *Krüppel* and *knirps* in the early *Drosophila* embryo

Martin Hülskamp, Christine Pfeifle & Diethard Tautz*

Institut für Genetik und Mikrobiologie, Universität München,
Maria-Ward-Str. 1a, 8000 München 19, FRG

SEGMENTATION of the *Drosophila* embryo depends on a hierarchy of interactions among the maternal and zygotic genes in the early embryo (see refs 1–3 for reviews). The anterior region is organized maternally by the *bicoid* (*bcd*) gene product, which forms a concentration gradient in the anterior half of the embryo^{4–6}. The gap genes are also involved in establishing the body plan, with *hunchback* (*hb*) being expressed both maternally and zygotically^{7,10}. Zygotic expression of *hb* is directly activated by the *bcd* gene product, leading to a subdivision of the embryo into an anterior half expressing zygotically provided *hb* protein and a posterior half that does not. A similar effect on maternally provided *hb* protein is caused by the gene *nanos*, which represses the translation of maternally provided transcripts in the posterior half. This regulation of *hb* protein is a prerequisite for abdomen development, because the presence of *hb* protein in the posterior half represses posterior segmentation^{14,17,18}. This repression mechanism suggests that posterior segmentation might not directly depend on maternal positional cues, but be solely organized at the zygotic level. Here we report further evidence to support this hypothesis and show that the *hb* protein itself is crucially involved in organizing abdominal segmentation. Differential concentrations of *hb* protein determine the anterior and posterior borders of expression of the gap gene *Krüppel* (*Kr*) and the anterior border of the gap gene *knirps* (*kni*), thus defining three positional values. These regulatory pathways are controlled in a redundant way, in part by *bcd* and in part by the maternal *hb* gene product.

Translation of maternally provided *hb* transcripts starts after the first few nuclear divisions, but is increasingly repressed in the posterior region so that at later stages it covers roughly the anterior half only. The initial expression of zygotically provided *hb* protein covers the same region, although it is much more abundant⁷. The high concentrations of *hb* protein provided by the zygotic *hb* (*hb_{zyg}*) can repress the transcription of *Kr*: the anterior *Kr* border shifts anteriorly in embryos mutant for *hb_{zyg}*^{8,9} (Fig. 1c). One would expect to see the same effect in embryos maternally mutant for *bcd*, as these fail to express *hb_{zyg}*. In fact, the shift is more pronounced in these embryos⁹ (Fig. 1e), indicating that either *bcd* itself, or a *bcd*-dependent gene other than *hb* acts as a second repressor of *Kr*. Nonetheless, it seems likely that the anterior *Kr* border is primarily defined by high concentrations of *hb* protein and that the effect of *bcd* is essentially redundant.

Low levels of *hb* protein can activate *Kr* expression. Low levels of *hb* protein may be provided either maternally or zygotically, as the protein diffuses posteriorly from its primary site of synthesis. Both protein sources are functionally interchangeable, as genetic removal of either *hb_{zyg}* or maternal *hb* (*hb_{mat}*) does not greatly affect the level of *Kr* transcription. But removal of both *hb_{zyg}* and *hb_{mat}* reduces *Kr* expression considerably (Fig. 1; compare *a* with *g*). This reduction of *Kr* expression also has phenotypic consequences; embryos double mutant for *hb_{zyg}* and *hb_{mat}* have a stronger phenotype than embryos mutant for *hb_{zyg}* alone¹⁰ (Fig. 1h). Double mutants lack head and thoracic seg-

ments, which is typical of the *hb_{zyg}* phenotype¹⁰, and abdominal segments A1–A3, whose normal development depends on the presence of *Kr*. The residual *Kr* expression in these embryos is caused by *bcd* activity; embryos mutant for both *bcd* and *hb_{mat}* (and *hb_{zyg}* because of the lack of *bcd*) fail to express *Kr*, as shown from *in situ* hybridization (Fig. 1i) and from the phenotypes (Fig. 1j, k). These embryos lack all *Kr*-dependent segments as far as A5 and have a perfect double abdomen with a mirror-image symmetry in A6. This phenotype is reminiscent of that of maternal *BicaudalD* (*BicD*) mutants¹¹, in which both *bcd* and *hb_{mat}* are repressed at the anterior¹², owing to the ectopic expression of posterior signals¹³. Therefore, the genetic removal of these two components produces the same effect. There is, however, one difference: *BicD* mutant embryos tend to have more abdominal segments. This can be explained by the fact that although *hb_{mat}* is repressed in both poles in *BicD* mutant embryos, it can be present in the middle of the embryo¹². There it could activate *Kr*, which in turn would specify the additional abdominal segments. We have indeed observed *Kr* expression at early stages in these embryos (data not shown), although *Kr* expression is usually absent at late stages¹².

The activation of *Kr* by *hb* seems to depend on a threshold level of the *hb* protein. Comparison of the early and late *Kr* domains of expression in embryos maternally mutant for *bcd* or mutant for *hb_{zyg}* shows that the early domain is much broader than the late one (Fig. 1c, d and e, f). It seems that the posterior border of *Kr* is initially set at the correct position, but moves anteriorly at later stages. This is consistent with *hb_{mat}* expression, which is gradually lost posteriorly at later stages⁷. Conversely, the *Kr* expression domain broadens posteriorly if *hb* is ectopically expressed in the posterior region. Such ectopic expression occurs in *oskar* mutant embryos, which fail to repress expression of *hb_{mat}* in the posterior region⁷, or can be experimentally induced by expressing *hb* from a heat-inducible promoter¹⁴. The effect on the posterior *Kr* border is the same in both cases: it expands posteriorly by about 10% (Fig. 1l, m), indicating that the *hb*-dependent activation of *Kr* is crucial for determining the normal posterior border of *Kr*. Under these artificial conditions the new border is determined by repression from the terminal system⁹.

Differential concentrations of *hb* protein are also involved in determining the anterior *kni* border. The transcription of *kni* is enhanced by low levels of *Kr* protein¹⁵ which diffuse across the *kni* expression domain, although this regulation is not required for determining the spatial limits of *kni* expression. We have compared *kni* expression in embryos lacking *hb_{zyg}* expression but containing either 0, 1 or 2 copies of *hb_{mat}*. If *hb_{mat}* is absent, *kni* expression expands anteriorly and forms a homogeneous domain between 35 and 65% of egg length (EL; 0% is the posterior end) (Fig. 2b). The new anterior border of *kni* may now be determined by *tailless* (*tll*), as *tll* represses *kni* expression¹⁵. If one dose of *hb_{mat}* is present expression of *kni* expands in a similar way, although the anterior part of the domain shows weaker expression than the posterior (Fig. 2c), indicating that the residual *hb_{mat}* still has some effect. This anterior expansion of *kni* is not seen when two doses of *hb_{mat}* are present in the absence of *hb_{zyg}*. Such a situation is found in *bcd* mutant embryos. No significant expansion of *kni* is visible under these conditions (Fig. 2d), although the whole domain follows the general shifts in the fate map seen in these embryos⁶. These observations suggest that medium levels of *hb* protein are required to set the anterior *kni* border.

The differential effect of *hb* protein concentration on the expression of *kni* and *Kr* probably explains why the anterior *kni* border lies anterior to the posterior *Kr* border in wild-type embryos (see also Fig. 4). To elucidate further this differential regulation, we created a situation where *hb* protein concentration would be too low to repress *kni*, but sufficiently high to activate *Kr*. This situation is seen in embryos maternally mutant for *bcd* and which have received only one dose of *hb_{mat}*. Under

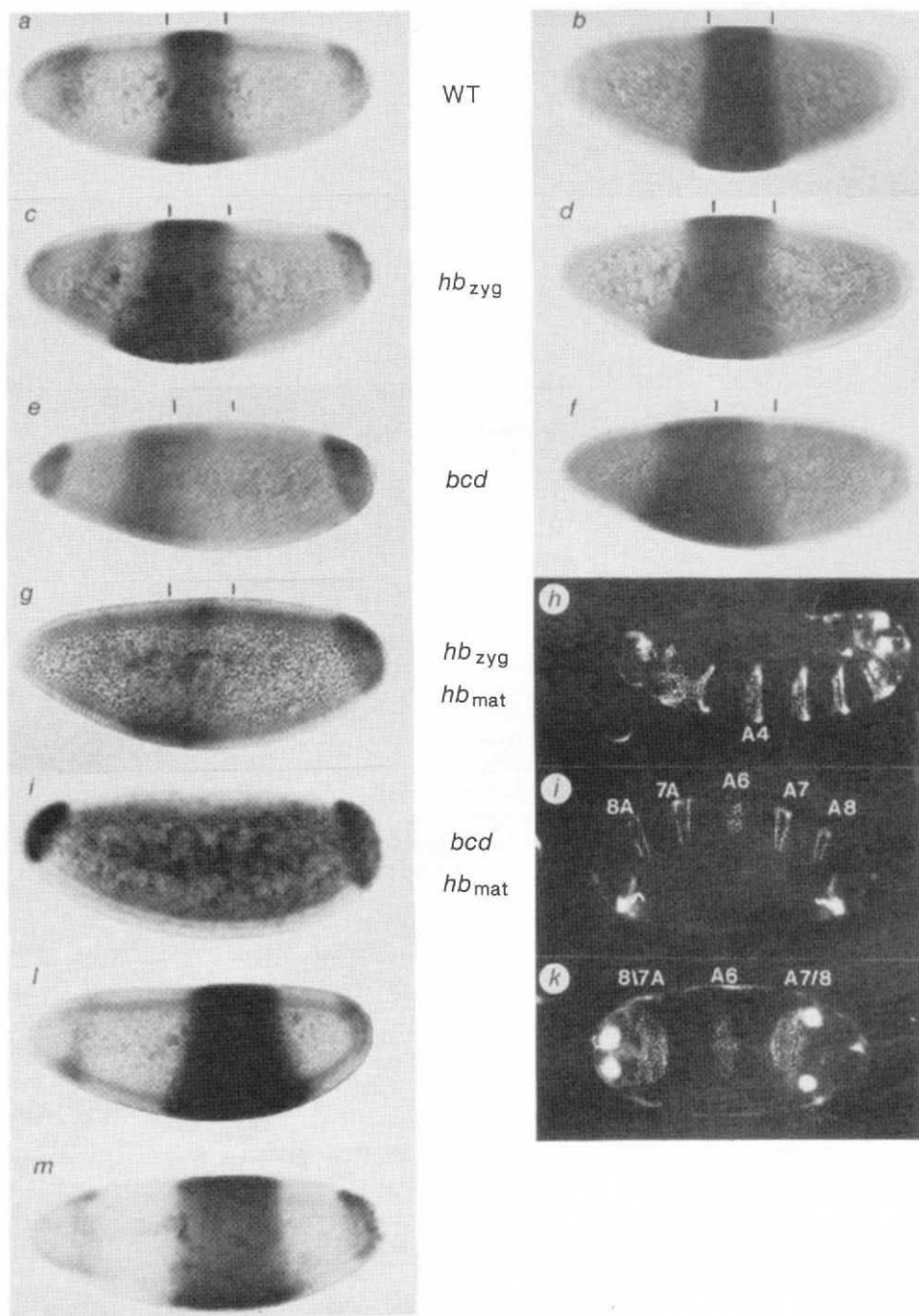
* To whom correspondence should be addressed.

these conditions, *Kr* activation is completely dependent on *hb* and is shifted anteriorly, as no repression occurs. The expression of *kni* covers the *Kr* expression domain completely (Fig. 3), suggesting that *hb* no longer represses. Thus, the different regulatory effects of *hb* can be separated by manipulating the *hb* protein concentration.

FIG. 1 Regulatory effects of *hb* and *bcd* on *Kr*. Whole-mount *in situ* hybridizations with the *Kr* probe⁸, and cuticle preparations are shown. The embryos to the left are all at late blastoderm stage, the embryos to the right (top) are early stages. Bars indicate in each case the position of the wild-type (WT) expression domain of *Kr*. *a, b*, *Kr* expression in wild-type embryos, late and early blastoderm respectively. *c, d*, Embryos zygotically mutant for *hb*^{9Q} (ref. 10) late and early blastoderm respectively. *e, f*, Embryos maternally mutant for *bcd*^{E1} (ref. 4) late and early blastoderm, respectively. *g, h*, Embryos maternally and zygotically mutant for *hb*^{9Q} (ref. 10); note that the central *Kr* domain of the embryo in *g* stains relatively weaker than the one of the embryo shown in *a*, as compared with the staining intensities of the posterior domains. *i, j*, Embryos maternally mutant for *hb*^{FB92} (ref. 10) and *bcd*^{E1}. Note the complete absence of *Kr* staining and the duplication of the posterior *Kr* domain, which is typical of *bcd* mutant embryos^{4,7,9}. *Kr* expression was not detected in earlier embryos (data not shown), in spite of overstaining. The cuticle phenotype in *j* shows a mirror-image duplication of abdominal segments, which is centred around abdominal segment A6. *k*, Embryo as in *j*, but in addition zygotically mutant for *hb*^{9Q}. This leads to a fusion of abdominal segments A7–A8, characteristic of the *hb* phenotype¹⁰. *l, m*, Posterior expansion of *Kr* expression in embryos ectopically expressing *hb* in the posterior region. *l*, Embryo maternally mutant for *osk*¹⁶⁶ (ref. 13). *m*, Embryo expressing *hb* under control of a heat-shock gene promoter¹⁴.

METHODS. Whole-mount *in situ* hybridizations were performed as described previously²¹. Genotypes of embryos in *g–k* can be obtained only by pole-cell transplantation experiments. These were essentially set up as described previously¹⁰, using embryos carrying the dominant female sterile *OvoD* mutation²² as acceptors and the *hb* or *hb/bcd* double mutant stocks as donors. In the experiments for the *hb* mutant germ lines we recovered 11 fertile females, which were mated with *hb* heterozygous males. Four females produced embryos, which showed the typical *hb*_{mat}/*hb*_{zyg} mutant phenotype¹⁰. The four females laid a total of ~400 eggs, of which ~200 were collected in 4-h periods, to obtain all stages up to late blastoderm. They were divided into four parts and used for *in situ* hybridizations and antibody staining with *Kr* and *kni* probes. These embryos were mixed with embryos from homozygous mutant *osk* females, which served as internal controls and as 'carriers' as they can be recognized because of their lack of pole cells¹³. In the experiments for the *hb/bcd* mutant germline, we recovered 12 fertile females, which were mated with *hb* heterozygous males. Five females produced embryos displaying the 'double abdomen' phenotype, whereas the other females produced embryos displaying the zygotic *hb* mutant phenotype. The number

An outline of the regulatory effects described above is shown in Fig. 4. The *hb* gene product is involved in setting at least three positional values in the embryo, namely the anterior *Kr* border, the anterior *kni* border and the posterior *Kr* border (see Fig. 4 legend). This positional information depends on different levels of the *hb* gene product, suggesting that *hb* forms a classical



of embryos analysed was similar to that for the above experiment. For the expression of *hb* under the control of a heat-shock gene promoter, we used *HSP70/hb* stock as described previously¹⁴. For the heat-shock experiments, embryos were collected on apple-juice agar plates at 25 °C for 30 min, aged for 90 min and heat-shocked at 37 °C for 30 min by transferring them onto pre-warmed agar plates. The embryos were then returned to 25 °C for 45 min before fixation. The success of the heat shock was always monitored by staining some of the embryos with anti-*hb* antibodies. All heat-shock experiments were also done in parallel with embryos that did not carry the *HSP70/hb* construct to ensure that the observed effects are solely due to the ectopically expressed *hb* protein.

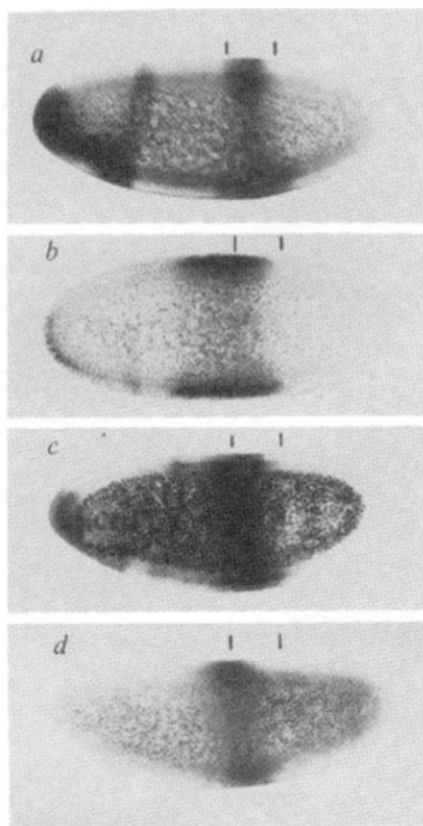


FIG. 2 Differential repression of *kni* by *hb*. Whole-mount *in situ* hybridizations with the *kni* probe²³ on late blastoderm stage embryos. Bars indicate the position of the primary *kni* expression domain in wild-type embryos. *a*, Wild-type embryo. *b*, Embryo maternally and zygotically mutant for *hb*⁹⁰. *c*, Embryo zygotically mutant for *hb*⁹⁰. *d*, Embryo maternally mutant for *bcd*^{E1}. The genotypes of the embryos in *b*, *c* and *d* are equivalent to 0, 1 and 2 doses of *hb*_{mat} in the absence of *hb*_{zyg}. METHODS. *In situ* hybridization and pole-cell transplantations were done as described in Fig. 1.

morphogenetic gradient. The figure also illustrates the redundant specification of the *Kr* borders by the *bcd* morphogenetic gradient.

Our results support previous speculations²⁷. Furthermore, cytoplasmic transplantation experiments have shown that the polarity of the abdomen can be determined at the gap-gene level¹⁶. But *hb* plays a special part among the gap genes—it transmits both the information from the posterior maternal system through its own maternal expression, and that from the

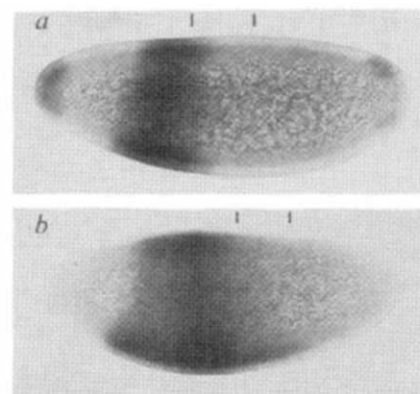
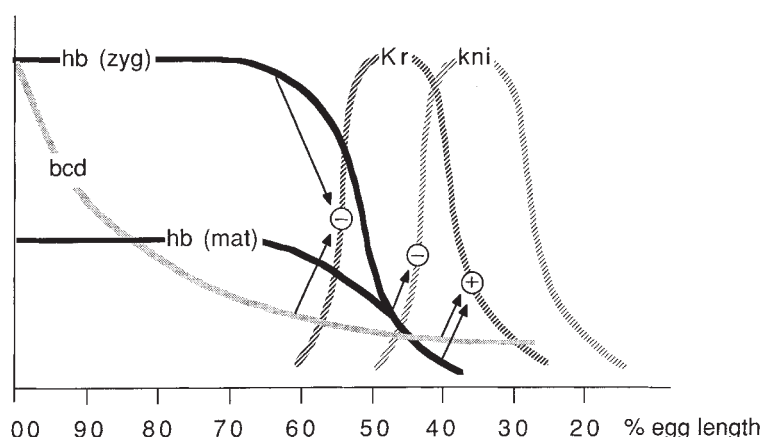


FIG. 3 Embryos maternally mutant for *bcd* and heterozygous for *hb*_{mat}, stained with *Kr* (*a*) and *kni* (*b*) probes by whole-mount *in situ* hybridization. Bars indicate the wild-type expression domains. The *Kr* and *kni* domains are shifted anteriorly and overlap completely under these conditions. This effect is variable and most penetrant at 29 °C, although it also occurs at lower temperatures.

anterior maternal system through its early zygotic expression. Both regulatory events lead to a subdivision of the embryo, providing it with a basic anterior-posterior polarity. The maternal *hb* expression is then involved in setting both the anterior *kni* border and the posterior *Kr* border, whereas the zygotic expression sets the anterior *Kr* border. So both aspects of *hb* expression have a defined function, although in *Drosophila* the zygotic function seems to be more important, because it can replace the maternal one in genetic experiments^{10,14,17,18}. This situation could be different in other insect systems; a higher level of maternal *hb* expression ought to produce the same regulatory effects as those provided by the early zygotic expression of *hb* in *Drosophila*. Such a reversal of relative importance could help explain some experimental results indicating an important role for the posterior organizing centre in other insect systems¹⁹.

We have not so far shown that any of the inferred interactions are direct, although this seems likely, at least for the action of *hb*. But the described interactions are certainly not the only ones that occur in the early embryo at the gap-gene level. It is increasingly evident that there are several redundant regulatory pathways specifying *Drosophila* development²⁰. We have discussed the apparent redundancy of the *hb*_{mat} expression and the redundant regulation of *Kr* by *hb* and *bcd*. Further redundant controls on the *Kr*-domain border are likely. In particular, we envisage a repression by *tll* (U. Gaul, personal communication) and a repression by *giant* (M. Levine, personal communication). The repression effect of *bcd* on *Kr* described above could in fact be mediated by this *giant* repression. Nonetheless, the

FIG. 4 Regulatory interactions of *hb* and *bcd* with *Kr* and *kni*. The primary expression domains of the different genes are roughly drawn to scale with respect to their position in the embryo. The different regulatory effects are symbolized by the arrows: ⊕, activation; ⊖, repression of the respective genes. The model shows that the anterior *Kr* border is defined by the repression effect of high concentrations of *hb* and *bcd* proteins, and the posterior *Kr* border is defined by the activating effect of low concentrations of these proteins. The anterior *kni* border is defined by the repression effect of medium to low levels of *hb* protein. The latter two regulatory events are specified by both zygotic and maternal expression of *hb*. Two other known regulatory mechanisms have been omitted from this diagram, namely the activating effect of *bcd* on *hb* (refs. 24–26)—which defines the posterior border of *hb* expression, and the repression effect of *tll* on *kni* expression (ref. 15)—which defines the posterior border of *kni* expression.



outline given in our model is sufficient to understand the logic of the system, even if additional redundant pathways are involved. Redundancy could be a prerequisite for developmental processes, ensuring that the complex flow of information necessary for the specification of body pattern is correctly transmitted. □

Received 19 March; accepted 18 May 1990.

1. Akam, M. *Development* **101**, 1–22 (1987).
2. Ingham, P. *Nature* **335**, 25–34 (1988).
3. Nüsslein-Volhard, C., Frohnhofer, H. G. & Lehmann, R. *Science* **238**, 1675–1681 (1987).
4. Frohnhofer, H. G. & Nüsslein-Volhard, C. *Nature* **324**, 120–125 (1986).
5. Driever, W. & Nüsslein-Volhard, C. *Cell* **54**, 83–93 (1988).
6. Driever, W. & Nüsslein-Volhard, C. *Cell* **54**, 95–104 (1988).
7. Tautz, D. *Nature* **332**, 281–284 (1988).
8. Jäckle, H., Tautz, D., Schuh, E., Seifert, E. & Lehmann, R. *Nature* **324**, 668–670 (1986).
9. Gaul, U. & Jäckle, H. *Cell* **51**, 549–555 (1987).
10. Lehmann, R. & Nüsslein-Volhard, C. *Dev Biol* **119**, 402–417 (1987).
11. Nüsslein-Volhard, C. *Roux's Arch. dev. Biol.* **183**, 249–268 (1977).
12. Wharton, R. P. & Struhl, G. *Cell* **59**, 881–892 (1989).
13. Lehmann, R. & Nüsslein-Volhard, C. *Cell* **47**, 141–152 (1986).
14. Struhl, G. *Nature* **338**, 741–744 (1989).
15. Pankratz, M., Hoch, M., Seifert, E. & Jäckle, H. *Nature* **341**, 337–340 (1989).
16. Lehmann, R. & Frohnhofer, H. G. *Development (Suppl.)* **107**, 21–29 (1989).
17. Hülkamp, M., Schröder, C., Pfeifle, C., Jäckle, H. & Tautz, D. *Nature* **338**, 629–632 (1989).
18. Irish, V., Lehmann, R. & Akam, M. *Nature* **338**, 646–648 (1989).
19. Sander, K. *Adv. Insect Physiol.* **12**, 125–238 (1976).
20. Elkins, T., Zinn, K., McAllister, L., Hoffmann, F. M. & Goodman, C. S. *Cell* **60**, 565–575 (1990).
21. Tautz, D. & Pfeifle, C. *Chromosoma* **98**, 81–85 (1989).
22. Busson, D., Gans, M., Komitopoulou, K. & Masson, M. *Genetics* **105**, 309–325 (1983).
23. Nauber, U. et al. *Nature* **336**, 489–492 (1988).
24. Schröder, C., Tautz, D., Seifert, E. & Jäckle, H. *EMBO J.* **7**, 2881–2887 (1988).
25. Driever, W. & Nüsslein-Volhard, C. *Nature* **337**, 138–143 (1989).
26. Struhl, G., Struhl, K. & Macdonald, P. *Cell* **57**, 1259–1273 (1989).
27. Struhl, G. *Ciba Foundn Symp.* **144** (eds Evered, D. & Marsh, J.) 65–86 (Wiley, Chichester, 1989).

ACKNOWLEDGEMENTS. We thank C. Nüsslein-Volhard and W. Driever for mutant stocks, especially the *bcd/hb* double mutant stock; G. Struhl for the HSP70/*hb* stock; U. Nauber and H. Jäckle for molecular probes; H. Jäckle, M. Pankratz, W. Lukowitz, R. Sommer and D. Weigel for stimulating discussions, and A. Beermann for the artwork. This work was supported by the Deutsche Forschungsgemeinschaft.

Stepwise phosphorylation of *myo*-inositol leading to *myo*-inositol hexakisphosphate in *Dictyostelium*

L. R. Stephens & R. F. Irvine

Biochemistry Department, AFRC Institute of Animal Physiology & Genetics Research, Cambridge Research Station, Babraham Hall, Cambridge CB2 4AT, UK

ALTHOUGH *myo*-inositol hexakisphosphate (InsP₆; phytate) is the most abundant inositol phosphate in nature¹ and probably has a wide variety of functions^{2–8}, neither the route of its synthesis from *myo*-inositol nor its metabolic relationships with other inositol-containing compounds (such as the second messenger inositol 1,4,5-trisphosphate, Ins(1,4,5)P₃) are known⁹. Here we report that the pathway by which InsP₆ is synthesized in the cellular slime mould *Dictyostelium*, and in cell-free preparations derived from them, is catalysed by a series of soluble ATP-dependent kinases independently of the metabolism of both phosphatidylinositol and Ins(1,4,5)P₃ (refs 10, 11). The intermediates between *myo*-inositol and InsP₆ are Ins3P, Ins(3,6)P₂, Ins(3,4,6)P₃, Ins(1,3,4,6)P₄ and Ins(1,3,4,5,6)P₅. The 3- and 5-phosphates of InsP₆ take part in futile cycles in which Ins(1,2,4,5,6)P₅ and Ins(1,2,3,4,6)P₅ are rapidly formed by dephosphorylation of InsP₆, only to be rephosphorylated to yield their precursor.

The cellular slime mould *Dictyostelium* contains 1 mM InsP₆ (ref. 12) and readily incorporates exogenous [³H]inositol ([³H]Ins) into an InsP₆-like compound¹³. We confirmed this and established that the highly polar ³H-labelled compound is indeed [³H]InsP₆, and noted the very low levels of [³H]InsP₂–

[³H]InsP₅ compounds that could be detected. In the presence of ATP-regenerating systems, homogenates of *Dictyostelium* (and the 100,000g supernatants derived from them) also rapidly incorporated [³H]Ins into [³H]InsP₆ (Fig. 1). The patterns of accumulating tritiated inositol phosphates in both intact *Dictyostelium* and their homogenates were very similar. Those that accumulated in the homogenates incubated with [³H]Ins were identified as [³H]Ins(3)P, [³H]Ins(3,6)P₂, [³H]Ins(3,4,6)P₃, [³H]Ins(1,3,4,6)P₄, [³H]Ins(1,3,4,5,6)P₅, [³H]Ins(1,2,3,4,6)P₅ and [³H]Ins(1,2,4,5,6)P₅ (see Fig. 1 legend). All of these ³H-inositol phosphates could be detected in [³H]Ins-labelled intact *Dictyostelium* (although the levels of [³H]Ins(3,6)P₂ and [³H]Ins(3,4,6)P₃ were so low that they were only characterized as ³H-compounds that were substrates for enzymes in *Dictyostelium* homogenates making [³H]InsP₆; L.R.S. and R.F.I., unpublished data).

A series of experiments were performed to determine first the substrate specificity of the initial step in the pathway, and second, which, if any, [³H]*myo*-inositol phosphates (including those purified from the lysates described above) are precursors of [³H]InsP₆ in *Dictyostelium*.

First, a soluble enzyme catalysing the phosphorylation of [³H]*myo*-inositol to [³H]Ins(3)P (Fig. 1 legend) was partially purified from *Dictyostelium* (L.R.S. and R.F.I., unpublished data) to enable the selectivity of the inositol binding site at the start of the pathway to be established: *neo*, (1*L*)-*chiro*, (1*D*)-*chiro*, *epi*, *muco*, *allo* and *scyllo*-inositol were not substrates. We did not test *cis*-inositol, although the structural overlap offered by the above results strongly suggests that it would not have been recognized as a substrate. Second, HPLC-purified [³H]*myo*-inositol phosphates either from *Dictyostelium* homogenates incubated with ATP and ³H-labelled Ins (as above; Fig. 1), or from alternative fully characterized sources, were incubated individually with *Dictyostelium* homogenates in the presence of ATP-regenerating systems. All of the lysate-derived [³H]*myo*-inositol phosphates listed above were rapidly and, if the time of incubation and concentration of homogenate were appropriate, quantitatively phosphorylated to [³H]InsP₆. But most of the alternatively derived 'standard' inositol phosphates were not phosphorylated in the cell-free *Dictyostelium* preparations (in each case the assays contained an internal positive control of an inositol phosphate contrastingly labelled with either ³H, ¹⁴C or ³²P and a substrate in the pathway defined above). The following inositol phosphates were not phosphorylated: [¹⁴C]Ins(2)P, [¹⁴C]Ins(4)P, [³²P]Ins(5)P, [³H]Ins(1)P, [³H]Ins(1,4)P₂, [³H]Ins(1,3)P₂, [³H]Ins(3,4)P₂, [³H]Ins(1,3,4)P₃, [³H]Ins(1,4,5)P₃ (confirms ref. 11), [³H]Ins(1,3,4,5)P₄, [³H]Ins(3,4,5,6)P₄ and D/L-[³H]Ins(1,2,3,4,5)P₅.

The following inositol phosphates (in addition to those purified from *Dictyostelium* supernatants; Fig. 1) were phosphorylated to InsP₆: [¹⁴C]Ins(3)P, [³H]Ins(3)P (the product of an inositol kinase partially purified from *Dictyostelium*, see above), [³H]Ins(1,3,4,6)P₄, [³H]Ins(1,3,4,5,6)P₅ and [³H]Ins(1,2,3,4,6)P₅ (see refs 14 and 15). When D/L-[³H]Ins(1,2,4,5,6)P₅ (a 1:1 mixture of [³H]Ins(1,2,4,5,6)P₅ and [³H]Ins(2,3,4,5,6)P₅; ref. 14) was incubated with *Dictyostelium* homogenates, a maximum of 48–51% could be phosphorylated (compared with the complete conversion of [³H]Ins(1,2,4,5,6)P₅ derived from [³H]Ins-prelabelled intact amoebae, or from *Dictyostelium* homogenates incubated with [³H]Ins and ATP (see above), or from [³H]InsP₆ incubated in *Dictyostelium* homogenates in the absence of exogenous ATP; see Fig. 1 and Tables 1 and 2).

Taken together, these results imply that Ins(1,3,4,6)P₄ is an intermediate in InsP₆ synthesis, and that it is derived from *myo*-inositol by way of Ins(3)P, Ins(3,6)P₂ and Ins(3,4,6)P₃. But the presence of three InsP₅ compounds both in intact amoebae and cell-free assays, all of which could be phosphorylated to InsP₆, made the characterization of the last two stages in the synthesis of InsP₆ more difficult. The half-times for the

conversion of $\text{Ins}(1,2,4,5,6)\text{P}_5$, $\text{Ins}(1,2,3,4,6)\text{P}_5$ and $\text{Ins}(1,3,4,5,6)\text{P}_5$ to InsP_6 in the intact cell, estimated by extrapolating results from *in vitro* assays (operating under first-order conditions with respect to the $[\text{H}]\text{InsP}_5$ s), were 0.8, 6.4 and 25 s respectively. Thus, it is the InsP_5 isomer that cannot be produced by a single-step phosphorylation of $\text{Ins}(1,3,4,6)\text{P}_4$, which is most rapidly converted to InsP_6 .

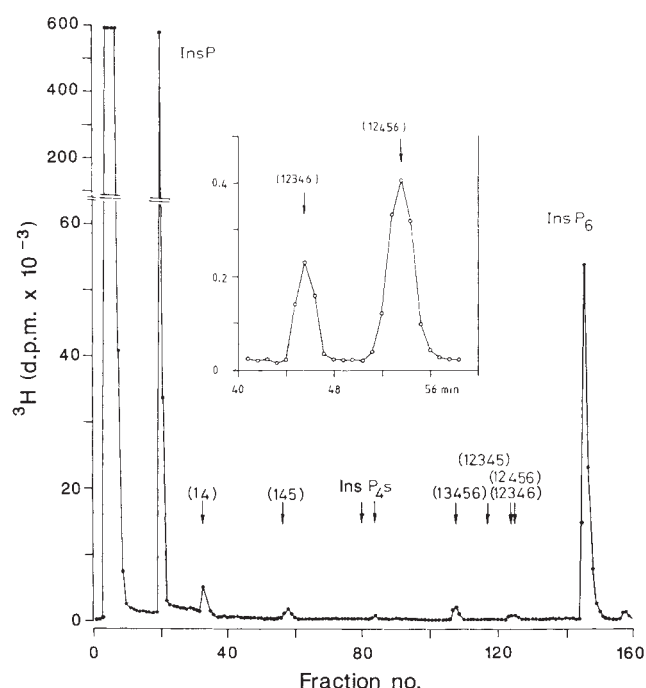
In assays in which $[\text{H}]\text{InsP}_6$ was incubated with *Dictyostelium* homogenates (or with essentially the same results, a 100,000g supernatant), however, in the presence or absence of ATP, $\text{Ins}(1,2,3,4,6)\text{P}_5$ and $\text{Ins}(1,2,4,5,6)\text{P}_5$, but not $\text{Ins}(1,3,4,5,6)\text{P}_5$, could be derived from the dephosphorylation of InsP_6 (Table 1). Moreover, when intact *Dictyostelium* were labelled for 3 h with $[\text{H}]\text{Ins}$, followed by extraction and HPLC-purification of $[\text{H}]\text{InsP}_6$ and $[\text{H}]\text{InsP}_5$ s, then both $[\text{H}]\text{Ins}(1,2,4,5,6)\text{P}_5$ and $[\text{H}]\text{Ins}(1,2,3,4,6)\text{P}_5$ had specific radioactivities significantly lower than $[\text{H}]\text{InsP}_6$ (Table 2), whereas that of $[\text{H}]\text{Ins}(1,3,4,5,6)\text{P}_5$ was significantly higher than $[\text{H}]\text{InsP}_6$. Together, these data suggest that the main precursor in the *de novo* synthesis of InsP_6 both *in vivo* and *in vitro* is $\text{Ins}(1,3,4,5,6)\text{P}_5$. $\text{Ins}(1,2,4,5,6)\text{P}_5$ and $\text{Ins}(1,2,3,4,6)\text{P}_5$ are likely to result from the phosphorylation of InsP_6 (as shown in homogenates above). $\text{Ins}(1,2,3,4,6)\text{P}_5$ could possibly be formed slowly by the phosphorylation of the 2-OH of $\text{Ins}(1,3,4,6)\text{P}_4$, but the results of experiments in which the temporal progress

of $[\text{H}]\text{Ins}(1,3,4,6)\text{P}_4$ phosphorylation in *Dictyostelium* homogenates was followed, showed that the only $[\text{H}]\text{InsP}_5$ that could be detected before all of the substrate had been consumed was $\text{Ins}(1,3,4,5,6)\text{P}_5$ (L.R.S. and R.F.I., unpublished data). Furthermore, the only detectable product of a partially purified preparation of $\text{Ins}(1,3,4,6)\text{P}_4$ kinase (from *Dictyostelium* cytosol and containing no $\text{Ins}(1,3,4,5,6)\text{P}_5$ 2-OH kinase activity; L.R.S. and R.F.I., unpublished data) was $\text{Ins}(1,3,4,5,6)\text{P}_5$, implying that $\text{Ins}(1,2,3,4,6)\text{P}_5$ is also derived by dephosphorylation of InsP_6 .

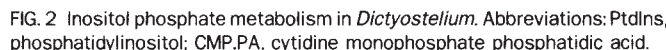
Our data allow the construction of a metabolic map (Fig. 2). This is the first report of an authentic inositol kinase activity other than that in a higher plant and has established that this enzyme is the route by which inositol can enter InsP_6 . The presence of an inositol 3-phosphate synthase activity in *Dictyostelium* (data not shown) suggests that some inositol 3-phosphate may be derived directly from glucose 6-phosphate, although the contribution that the flux through this enzyme makes to the turnover of $\text{Ins}(3)\text{P}$ is unclear. The independence of this pathway from $\text{Ins}(1,4,5)\text{P}_3$ metabolic intermediates in *Dictyostelium* (note the absence of $\text{Ins}(1,4,5)\text{P}_3$ -3-kinase¹¹; L.R.S. and R.F.I., unpublished data) allows the synthesis of InsP_6 to be regulated separately from that of $\text{Ins}(1,4,5)\text{P}_3$ and the entire inositol phospholipid family. The ability, however, of many mammalian cells to generate $\text{Ins}(3)\text{P}$, $\text{Ins}(1,3,4,6)\text{P}_4$ and $\text{Ins}(1,3,4,5,6)\text{P}_5$ from $\text{Ins}(1,4,5)\text{P}_3$ (refs 10, 15) implies that if

FIG. 1 Anion-exchange HPLC separation of the ^3H -labelled products formed in a *Dictyostelium* homogenate in the presence of $[\text{H}]\text{Ins}$ and an ATP-regenerating system.

METHODS. NC4 (a laboratory strain of *Dictyostelium discoideum*) were co-cultured with *Klebsiella aerogenes* as previously described¹⁸. When plates were half-cleared the amoebae were collected into 20 mM KCl, 20 mM NaCl, 1 mM CaCl_2 , 10 mM 2-[N-morpholino]-ethanesulphonic acid (to pH 6.2 with NaOH; NS-MES buffer) and freed from bacteria by differential centrifugation¹⁸. The amoebae were finally resuspended into ice-cold 10% (v/v) glycerol, 10 mM HEPES, 1 mM EGTA, 0.2 mM phenylmethylsulphonyl fluoride, (pH 7.5, 4 °C), then lysed through a Whatman No. 3 filter paper¹⁹. Less than 1% of cells remained intact. Aliquots (150 μl) of the homogenate were incubated in 200- μl assays containing (final composition) 100 mM KCl, 5 mM ATP, 6 mM MgCl_2 , 25 mM HEPES, 1 mM EGTA, 10 mM creatine phosphate, 5 U ml^{-1} creatine phosphokinase, 1 mM DTT, 1 mg ml^{-1} BSA (pH 7.0, 25 °C) and 100 μCi $[\text{H}]\text{Ins}$ (Amersham). After 50 min at 25 °C, the assay was quenched with 600 μl 3.16% (v/v) perchloric acid and centrifuged at 15,000 r.p.m. (2 min), the supernatant was removed and added immediately to 200 μl 2 M KOH, 0.1 M MES and 50 μl 0.1 M EDTA (pH 7.0, 25 °C). The resulting solution was confirmed to be about pH 6. ^{32}P - and ^{14}C -labelled standards were then added (their elution positions are indicated; the InsP_4 s, in order of increasing retention time, were $\text{Ins}(1,3,4,5)\text{P}_4$ and $\text{Ins}(1,3,4,6)\text{P}_4$). The sample was filtered, applied to a Partisphere SAX anion exchange column and eluted with a NaH_2PO_4 gradient (pH 3.75)¹⁴. Fractions were collected every 30 s and, except for those containing $\text{Ins}(1,2,4,5,6)\text{P}_5$ and $\text{Ins}(1,2,3,4,6)\text{P}_5$, were counted for ^3H , ^{14}C and ^{32}P using standard liquid scintillation techniques. Fractions containing $\text{Ins}(1,2,4,5,6)\text{P}_5$ and $\text{Ins}(1,2,3,4,6)\text{P}_5$ were pooled, desalted and reappplied to a Partisphere WAX HPLC column, eluted with 0.35 M $(\text{NH}_4)_2\text{HPO}_4$ (pH 3.2 with H_3PO_4) and the resolved isomers finally quantified by liquid scintillation counting (see inset)¹⁴. $[\text{H}]\text{Ins}$ -labelled peaks were identified as follows: (1) $[\text{H}]\text{Ins}(3)\text{P}$ co-migrated with $\text{D/L-Ins}(1)\text{P}$ but not $\text{Ins}(2)\text{P}$, $\text{Ins}(4)\text{P}$ or $\text{Ins}(5)\text{P}$ during anion-exchange chromatography¹⁵, yielded $[\text{H}]\text{L-glucitol}$ upon partial periodate-oxidation, reduction and dephosphorylation¹⁴ and served as a substrate for a soluble $\text{Ins}(3)\text{P}$ kinase activity isolated from *Dictyostelium* homogenates (data not shown); (2) $[\text{H}]\text{Ins}(3,6)\text{P}_2$ co-migrated with $\text{Ins}(1,4)\text{P}_2$ (and not $\text{Ins}(4,5)\text{P}_2$), lost all of its ^3H on complete periodate-oxidation and yielded $[\text{H}]\text{L-iditol}$ and $[\text{H}]\text{D-altritol}$ upon partial periodate-oxidation, reduction and dephosphorylation^{14,20}; (3) $[\text{H}]\text{Ins}(3,4,6)\text{P}_3$ eluted from a Partisphere SAX anion-exchange HPLC column significantly later than $\text{Ins}(1,4,5)\text{P}_3$ and yielded $[\text{H}]\text{L-iditol}$ on periodate-oxidation, reduction and dephosphorylation; (4) $[\text{H}]\text{Ins}(1,3,4,6)\text{P}_4$ co-migrated with $[\text{H}]\text{Ins}(1,3,4,6)\text{P}_4$ but not with $\text{Ins}(1,3,4,5)\text{P}_4$, $\text{Ins}(1,2,4,5)\text{P}_4$ or $\text{Ins}(2,3,4,6)\text{P}_4$ ($[\text{H}]\text{Ins}(2,3,4,6)\text{P}_4$ was prepared as described¹⁴, and eluted substantially later than $\text{Ins}(1,3,4,6)\text{P}_4$ from standard Partisphere SAX-anion exchange HPLC columns; $[\text{H}]\text{Ins}(1,2,4,5)\text{P}_4$ was prepared by treating $[\text{H}]\text{Ins}(1,3,4,5)\text{P}_4$ with 1.0 M HCl at 80 °C for 8 min, and eluted from a Partisphere SAX anion-exchange HPLC column after $\text{Ins}(1,3,4,5)\text{P}_4$ and $\text{Ins}(1,3,4,6)\text{P}_4$ but before $\text{Ins}(2,3,4,5)\text{P}_4$ and $\text{Ins}(2,3,4,6)\text{P}_4$),



yielded $[\text{H}]\text{Ins}$ on periodate-oxidation (0.1 M periodic acid, pH 2.0), reduction and dephosphorylation, and gave a single additional $[\text{H}]\text{InsP}_4$ when treated with 1.0 M HCl at 80 °C for 8 min that eluted at precisely the time expected for $[\text{H}]\text{Ins}(2,3,4,6)\text{P}_4$ and that yielded $[\text{H}]\text{Ins}$ on periodate-oxidation (0.1 M periodic acid, pH 2.0), reduction and dephosphorylation; (5) $[\text{H}]\text{Ins}(1,3,4,5,6)\text{P}_5$ co-migrated with $[\text{H}]\text{Ins}(1,3,4,5,6)\text{P}_5$ on both Partisphere SAX and WAX anion-exchange HPLC columns, but not with $\text{D/L-}[\text{H}]\text{Ins}(1,2,4,5,6)\text{P}_5$, $\text{D/L-}[\text{H}]\text{Ins}(1,2,3,4,5)\text{P}_5$ or $[\text{H}]\text{Ins}(1,2,3,4,6)\text{P}_5$ (see figure, and ref. 14); (6) $[\text{H}]\text{Ins}(1,2,3,4,6)\text{P}_5$ ran appropriately relative to internal standards in the HPLC protocol described above; (7) $[\text{H}]\text{Ins}(1,2,4,5,6)\text{P}_5$ ran appropriately relative to internal standards in the HPLC protocol described above and when enzymatically dephosphorylated yielded $[\text{H}]\text{Ins}(1,2,6)\text{P}_3$ and $[\text{H}]\text{Ins}(1,2,5,6)\text{P}_4$ (as opposed to $[\text{H}]\text{Ins}(2,3,4)\text{P}_3$ or $[\text{H}]\text{Ins}(2,3,4,5)\text{P}_4$; data not shown, and ref. 14). Moreover, the $[\text{H}]\text{Ins}(1,2,4,5,6)\text{P}_5$ was completely converted to $[\text{H}]\text{InsP}_6$ by an $\text{Ins}(1,2,4,5,6)\text{P}_5$ 3-OH kinase activity present in *Dictyostelium* homogenates, whereas only 49% of the ^{32}P derived from an internal 'spike' of $\text{D/L-}[\text{H}]\text{Ins}(1,2,4,5,6)\text{P}_5$ was recovered in InsP_6 (see above and Table 2 legend).



There is already some evidence that at least part of the pathway described in *Dictyostelium* exists in mammalian cells. For example, although $[^3\text{H}]\text{Ins}(1,2,4,5,6)\text{P}_5$ and $[^3\text{H}]\text{Ins}(1,2,3,4,6)\text{P}_5$ have not been described in cell extracts before, they made up 0.5–2.0% of the total $[^3\text{H}]\text{InsP}_5$ derivatives detected in acid extracts of $[^3\text{H}]\text{Ins}$ -prelabelled mammalian cells (for example, HL60 and NG115-401L cells, where the predominant $[^3\text{H}]\text{InsP}_5$ in both was $[^3\text{H}]\text{Ins}(1,3,4,5,6)\text{P}_5$; L.R.S., D. Poyner

[³ H]Ins metabolite	% of total ³ H recovered		
	No enzyme blank	+ATP	-ATP
Ins	—	0.05	0.31
InsP	—	—	0.74
InsP ₂ s	—	—	9.3
InsP ₃ s	—	0.01	5.6
InsP ₄ s	—	0.13	13.7
InsP ₅ s	—	0.47	0.03
Ins(1,3,4,5,6)P ₅	—	0.48	3.8
Ins(1,2,3,4,6)P ₅	—	0.15	12.9
Ins(1,2,4,5,6)P ₅	—	0.15	12.9
InsP ₆	99.99	98.7	54.7

TABLE 2 Specific radioactivities of [^3H]InsP₅s and [^3H]InsP₆ in [^3H]Ins-labelled amoebae

Amoebae were labelled (3 h) with $[^3\text{H}]\text{Ins}$ (2×10^7 cells ml^{-1} , $40 \mu\text{Ci } [^3\text{H}]\text{Ins ml}^{-1}$, $2 \mu\text{Ci } ^{32}\text{P}_i \text{ ml}^{-1}$ (where P_i is inorganic phosphate) $1 \mu\text{g streptomycin ml}^{-1}$ in 50 ml NS-MES buffer, shaking at 180 r.p.m. and 22°C ; the cells were labelled with $^{32}\text{P}_i$ to facilitate the detection and isolation of the $[^3\text{H}]\text{Ins}[^{32}\text{P}]\text{P}_5\text{s}$, then pelleted by centrifugation. The medium was aspirated, and the cells quenched with 2 ml ice-cold 5% (v/v) perchloric acid. The samples were vortexed at 4°C (5 min), the cellular debris pelleted by centrifugation and the supernatant neutralized with 2M KOH, 0.1M MES, 25 mM EDTA. The three $[^3\text{H}]\text{Ins}[^{32}\text{P}]\text{P}_5\text{s}$ and $[^3\text{H}]\text{Ins}[^{32}\text{P}]\text{P}_6$ were resolved and collected by sequential use of Partisphere SAX and WAX anion-exchange HPLC columns (Fig. 1 legend). The HPLC-purified, desalted $[^3\text{H}]\text{inositol } [^{32}\text{P}]\text{phosphates}$ were totally dephosphorylated to free inositol using alkaline phosphatase, desalted with mixed-bed ion-exchange resin and redissolved in H_2O (ref. 20). The *myo*-inositol content of each sample was determined using an enzyme-linked fluorimetric assay²¹. The samples were incubated with *myo*-inositol dehydrogenase (Sigma) and the NADH formed used to drive the enzyme-catalysed (Diaphorase, Sigma) reduction of resazurin, which becomes intensely fluorescent in the process. Final sample fluorescence was measured in a Perkin-Elmer LS-5B luminescence spectrometer. This protocol was applied to samples that had been diluted to ensure they contained 0.8–1.8 nM *myo*-inositol (standards were 25–150 pmol per assay). The data are means $\pm$ s.e.m. ($n=15$) pooled from three independent determinations. The statistical significance (P) of the difference between the specific radioactivity of the $[^3\text{H}]\text{InsP}_5\text{s}$ and that of $[^3\text{H}]\text{InsP}_6$ is shown. The intracellular concentrations of these metabolites were calculated to be: InsP_6 , 0.594 mM; $\text{Ins}(1,3,4,5,6)\text{P}_5$, 8 μM ; $\text{Ins}(1,2,3,4,6)\text{P}_5$, 16.6 μM ; $\text{Ins}(1,2,4,5,6)\text{P}_5$, 36 μM (there was $\sim 115 \mu\text{l}$ intracellular volume in the original cell suspension).

Finally, the metabolic scheme defined above suggests that the experimental strategy currently employed to investigate inositol polyphosphate metabolism in most mammalian cells (that is, labelling to quasi-steady-state with [^3H]Ins and measuring the quantity of ^3H in the metabolite of interest) will give misleading results about the speed with which InsP_6 is metabolized, because the rate of labelling of [^3H]Ins P_6 with [^3H]Ins will be dominated by the progress of the tracer through the synthetic pathways, rather than by any reactions, however rapid, metabolizing the Ins P_6 itself. $\square$

1. Cosgrove, D. J. *Inositol Phosphates* (Elsevier, Amsterdam, 1980).
2. Vallejo, M., Jackson, T., Lightman, S. & Hanley, M. R. *Nature* **330**, 656-658 (1987).
3. Nicotietti, F., Bruno, V., Fione, L., Vavalloro, S. & Canonico, P. L. *J. Neurochem.* **53**, 1026-1030 (1989).
4. Barraco, R. A., Phyllis, J. W. & Simpson, L. L. *Eur. J. Pharmac.* **173**, 75-84 (1989).
5. Graf, E., Mahoney, J. R., Bryant, R. G. & Eaton, J. W. *J. biol. Chem.* **259**, 3620-3624 (1987).
6. Williams, S. G. *Pl. Physiol.* **45**, 376-381 (1970).
7. Biswas, S., Maity, I. B., Chakrabarti, S. & Biswas, B. B. *Arch. biochim. biophys. Acta* **185**, 557-566 (1978).
8. Biswas, S. & Biswas, B. B. *Biochim. biophys. Acta* **108**, 710-713 (1965).
9. Berridge, M. J. & Irvine, R. F. *Nature* **341**, 197-205 (1989).
10. Shears, S. B. *Biochem. J.* **260**, 313-324 (1989).

11. Van Haastert, P. J. M. *et al.* *Biochem. J.* **258**, 577–586 (1989).
12. Martin, J., Foray, M., Klein, G. & Satre, M. *Biochim. biophys. Acta* **931**, 16–25 (1987).
13. Europe-Finner, G. N. *et al.* *J. Cell Sci.* **89**, 13–20 (1988).
14. Stephens, L. R. in *Methods in Inositol Research* (ed. Irvine, R. F.) (Raven, New York, in the press).
15. Stephens, L. R., Hawkins, P. T., Barker, C. J. & Downes, C. P. *Biochem. J.* **253**, 721–723 (1988).
16. French, P. J., Bunce, C. M., Brown, G., Creba, J. A. & Michell, R. H. *Biochem. Soc. Trans.* **16**, 985–986 (1988).
17. Szwergold, B. S., Graham, R. A. & Brown, T. R. *Biochem. biophys. Res. Commun.* **149**, 874–881 (1987).
18. Kay, R. R. & Trevan, D. J. *J. Embryol. exp. Morph.* **62**, 369–378 (1981).
19. Das, O. P. & Henderson, E. J. *Biochem. biophys. Acta* **736**, 45–65 (1983).
20. Stephens, L. R. & Downes, C. P. *Biochem. J.* **265**, 435–452 (1990).
21. Maslanski, J. & Busa, W. B. in *Methods in Inositol Research* (ed. Irvine, R. F.) (Raven, New York, in the press).

ACKNOWLEDGEMENTS. We thank P. Hawkins for helpful discussions, R. Kay for advice about handling *Dictyostellium*, Profs S. Angyal, L. Anderson and N. Baggett for their generous gifts of a selection of inositols, and W. Busa and J. Maslanski for making their version of an inositol assay available to us. L.R.S. is a Babraham Research Fellow.

Autoregulation of *pit-1* gene expression mediated by two *cis*-active promoter elements

Ruoping Chen^{*†}, Holly A. Ingraham^{*‡},
Maurice N. Treacy^{*}, Vivian R. Albert^{*‡}, Laura Wilson^{*}
& Michael G. Rosenfeld^{*‡}

^{*}Eukaryotic Regulatory Biology Program, Center for Molecular Genetics, School of Medicine, M-013, La Jolla, California 92093, USA

[†]Biochemistry Program, University of California, Davis, California, USA

[‡]Howard Hughes Medical Institute, University of California, San Diego, La Jolla, California 92093, USA

THE *pit-1* gene is a member of a large family of genes that encode proteins which are involved in development and which contain a highly homologous region, referred to as the POU domain^{1–9}. Pit-1, a pituitary-specific transcription factor, can activate the transcription of the growth hormone and prolactin promoters^{1,10}. It is expressed in mature thyrotroph, somatotroph and lactotroph cell types^{11,12} of the anterior pituitary which arise sequentially during development; somatotrophs and lactotrophs, which secrete growth hormone and prolactin, respectively, are the last to arise¹³. Intriguingly, during ontogeny, *pit-1* transcripts are observed in the rat neural tube and neural plate (embryonic day 10–11) and disappear thereafter (day 13), only to reappear exclusively in the anterior lobe of the pituitary gland (day 15) just before activation of prolactin and growth hormone^{12,14,15}. This biphasic pattern suggests a complex mechanism of initial activation of *pit-1* gene expression. Transcription and transfection analyses *in vitro* using wild-type and mutated promoters indicate that Pit-1 can positively autoregulate the expression of the *pit-1* promoter as a consequence of binding to two Pit-1-binding elements. Mutation of the 5' Pit-1-binding site abolished positive autoregulation, whereas mutation of the element located immediately 3' of the cap site markedly increased expression of the *pit-1* promoter. These data are consistent with a positive, attenuated autoregulatory loop that seems to function in maintaining *pit-1* gene expression.

To begin to analyse the mechanisms of *pit-1* gene activation and expression, two overlapping clones containing the 5' flanking region of the *pit-1* gene were isolated from a rat genomic library, subcloned and sequenced (Fig. 1a); sequences corresponding to those from positions –211 to +120 are shown in Fig. 1a. The transcription initiation site was identified by RNase-A protection assay (Fig. 1b). Major and minor protected species were observed at positions corresponding to 170 and 175 nucleotides when using total RNA from rat pituitary cultured cell lines (GC), no bands were protected with total RNA obtained from HeLa or BJAB cells (human B cell) (Fig. 1b). Therefore, the transcription initiation site of the *pit-1* gene is located 120 base pairs (bp) upstream of the translation initiation codon, ATG.

The initial 200 bp of the promoter was sufficient to confer pituitary cell-specific gene expression (see below); we therefore studied this region of the promoter in more detail. The sequence of the *pit-1* promoter revealed a TATA sequence at –30 bp, two potential Pit-1-binding sites, PitB1 and PitB2 (Fig. 1a, boxed areas), with sequence similarity to the consensus sequence of (T/A) (T/A) TATNCAT (refs 16, 17), and two sequences (underlined) that could represent cyclic AMP response elements¹⁸. Indeed, bacterially expressed rat CREB protein¹⁸ bound to both of the putative CRE elements—the –191 to –200 palindromic CRE (data not shown), and the –137 to –163

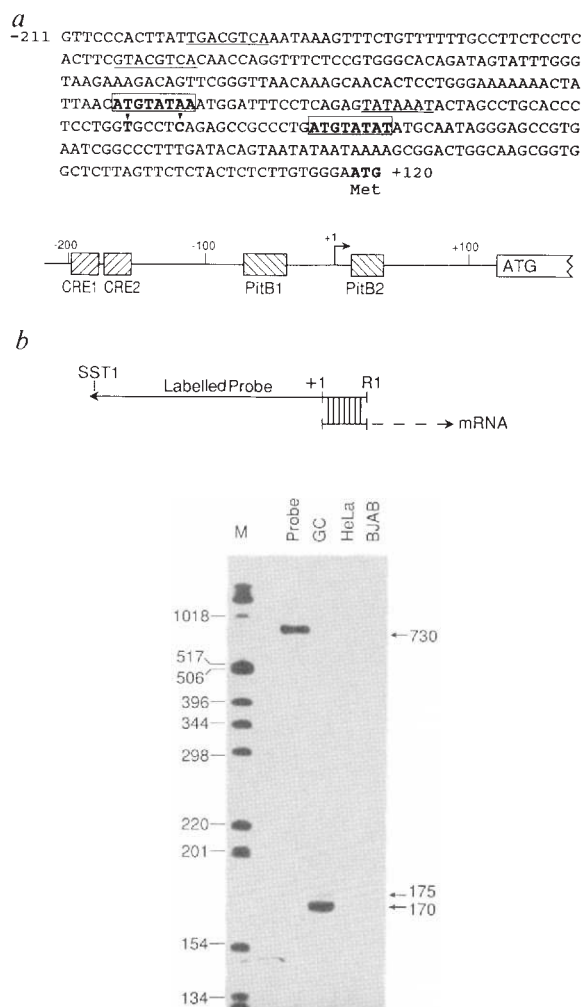


FIG. 1 Structure of the *pit-1* promoter region. *a*, Nucleotide sequences of the *pit-1* promoter region (–210 to +120); the major transcription start site is numbered +1 and corresponds to the position marked by the 3'-most arrow (C). The two sites containing a Pit-1-binding consensus (bold and boxed), two putative CRE sites (underlined), and the putative TATA box (underlined) are indicated. *b*, Transcriptional start site of the *pit-1* gene was mapped by a RNase-A protection assay. The size of full-length probe (730 bp) and protected species of 175 and 170 bp are indicated by arrows.

METHODS. A 26-residue oligonucleotide (5'-AAAGG-GCGAT-TCACG-GCTCC-CTATTG-3') derived from 5' untranslated sequences of the rat *pit-1* cDNA was used to screen²⁵ ~2 × 10⁵ independent recombinants from a rat GEM-11 genomic library, yielding two positive clones. A 3.8-kb *Hind*III fragment was subcloned and both strands were sequenced by the dideoxynucleotide method of Sanger²⁶. Total RNA (10 µg) was isolated from different cultured cell lines and analysed by RNase protection as previously described¹⁴. A 730-bp *Sst*I-*Eco*RI genomic fragment containing 150 bp of the 5' untranslated and partial coding sequences of *pit-1* cDNA was cloned into plasmid pSP65 (Promega) to generate antisense RNA probe. ³²P-end-labelled 1-kilobase ladder (BRL) was used to provide size markers (lane M, in bp).

sequence encompassing a GTACGTC sequence (Fig. 2b). Activation of cAMP-dependent protein kinase by dibutyl cAMP resulted in a 6- to 12-fold stimulation of expression of fusion genes under control of the *pit-1* promoter in GC cells (data not shown). Vascular invasion of the pituitary gland, with delivery of hypothalamic trophic factors occurs at embryonic day 16, in parallel with increased *pit-1* gene expression¹². This increased *pit-1* expression may be due to the predicted rise in cAMP levels.

Nuclear extracts from GC cell lines, which contain high levels of Pit-1 protein^{2,10,16}, conferred a pattern of DNase-I protection encompassing the two putative Pit-1-binding sites (Fig. 1a). The same results were obtained using a preparation of bacterially expressed Pit-1 (ref. 19) (Fig. 2a). In contrast, HeLa nuclear extracts did not protect the two Pit-1-binding sites from DNase I digestion, giving patterns comparable to those obtained in the absence of added nuclear extract (Fig. 2a).

To determine whether Pit-1 or other proteins were responsible for the observed binding in GC nuclear extracts, we performed gel shifts in the presence of preimmune serum or specific anti-Pit-1 sera. A highly specific Pit-1 antisera shifted virtually all of the GC nuclear extract/PitB1 complex and >90% of the GC nuclear extract/PitB2 complexes (Fig. 3a). Further, bacterial Pit-1 generated identical gel shift patterns with each Pit-1 *cis*-active element as did the GC nuclear extracts (Fig. 3a). Therefore, Pit-1 seems to be the predominant protein in GC crude nuclear extracts that binds the Pit-1 promoter elements. To investigate the affinity of Pit-1 for these elements, we performed competition analyses using the high-affinity Pit-1-binding site in the rat prolactin gene (Prl-1P), which exhibits a dissociation constant $K_D \approx 5 \times 10^{-10}$ M (ref. 19). Both the 5' (PitB1) and the 3' (PitB2) binding elements had a high affinity for Pit-1 protein (Fig. 3b). The high levels of Pit-1 protein in mature pituitary cells^{10,12} predict occupancy of both sites.

To test whether Pit-1 binding to elements in the *pit-1* promoter and 5' untranslated regions resulted in gene activation, reporter plasmids under control of various truncated *pit-1* promoters

were transfected into GC pituitary cells, or cotransfected with a plasmid containing a Pit-1 complementary DNA transcription unit into a heterologous cell line lacking endogenous Pit-1 (Fig. 4a, b). Expression of Pit-1 protein specifically activated the intact *pit-1* promoter in heterologous cells (CV-1), characteristically resulting in a 10- to 30-fold stimulation of reporter genes under control of the -210-bp *pit-1* promoter region (Fig. 4b). As confirmed by transcription *in vitro*, the correct cap site was used in all constructs (Fig. 4c). Inclusion of sequences further 5' of this region (-1.8 kilobases (kb)) resulted in no further Pit-1-dependent gene activation (data not shown). There was, however, an enhancer which was not cell-specific located between -850 and -1,800 bp, capable of exerting effects in all cell types tested. Promoters deleted to -80 bp exhibited a similar Pit-1-dependent induction, suggesting that the *pit-1* sequences located between -80 to +34 were alone responsible for the transcriptional stimulation observed (Fig. 4a, b). Mutation of the first Pit-1 site (mPitB1) to a site incapable of binding Pit-1, as determined by DNase-I footprinting or gel shift assays (data not shown), abolished Pit-1-dependent fusion gene expression in CV-1 cells, suggesting that the PitB1 site was critical for full Pit-1-dependent stimulation (Fig. 4b). Interestingly, mutation of the second Pit-1 binding site to a nonbinding element (mPitB2), in the context of the -210 bp promoter, resulted in a marked (five to seven times) increase in the ability of Pit-1 to stimulate reporter gene expression (Fig. 4b). The same pattern of functional effects was observed with *pit-1* fusion gene expression in GC pituitary cells (Fig. 4a). The actions of the PitB2 element seem to be position-dependent because when the element was placed in a novel context 5' of the prolactin gene promoter (-36 to +34), it conferred Pit-1-dependent transcriptional stimulation *in vitro* (data not shown).

The role of the Pit-1-binding sites was further investigated by transcription analysis *in vitro*. Fusion genes under control of the *pit-1* promoter (-210 to +34) were expressed at very low levels in GC pituitary nuclear extracts (Fig. 4c). Mutation of the PitB2 element resulted in strikingly increased expression (Fig. 4c). In

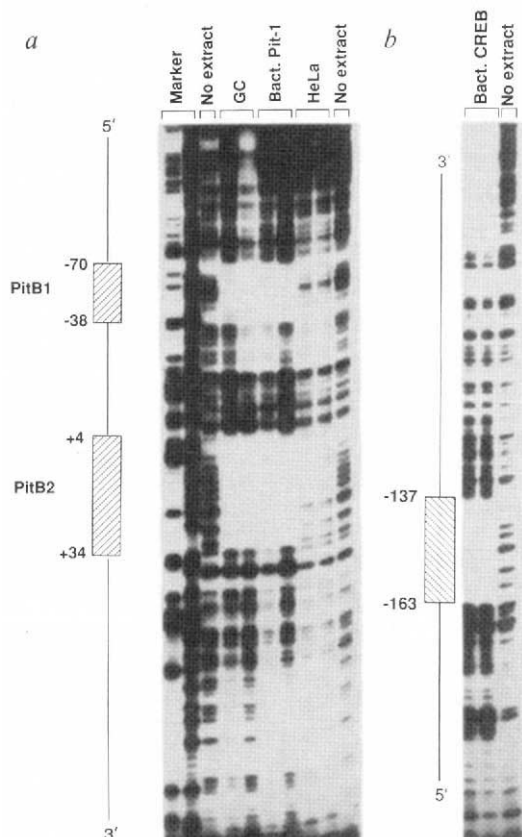


FIG. 2 Binding of Pit-1 and CREB proteins to the *pit-1* promoter. *a*, An end-labelled DNA probe containing 320 bp of the *pit-1* promoter (-210 to +110) was subjected to DNase I footprinting (two concentrations) using GC or HeLa cell nuclear extracts, or a denatured-renatured bacterially expressed Pit-1. Control experiments (no extract) and markers are shown. The two protected regions are indicated by boxes labelled as PitB1 and PitB2. *b*, Binding of bacterial CREB to a *pit-1* promoter probe (-210 to +110), using 3 μ l (left) and 8 μ l (right) of a partially purified bacterially expressed CREB protein. The site shown is the atypical CRE binding site (see *a*). METHODS. DNA probes containing the *pit-1* promoter region (-210 to +110) were generated by polymerase chain reaction. DNase-I footprinting reactions were performed as previously described¹⁰. About 75 μ g GC and 120 μ g HeLa-cell nuclear extracts or 1.5 μ g bacterial extracts containing Pit-1 or CREB were used. DNA markers were generated using G and A + G sequencing ladders.

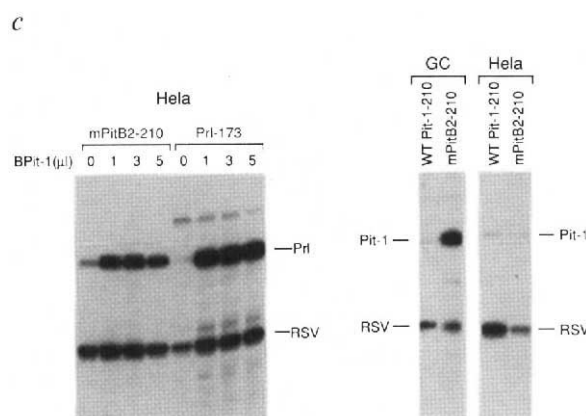
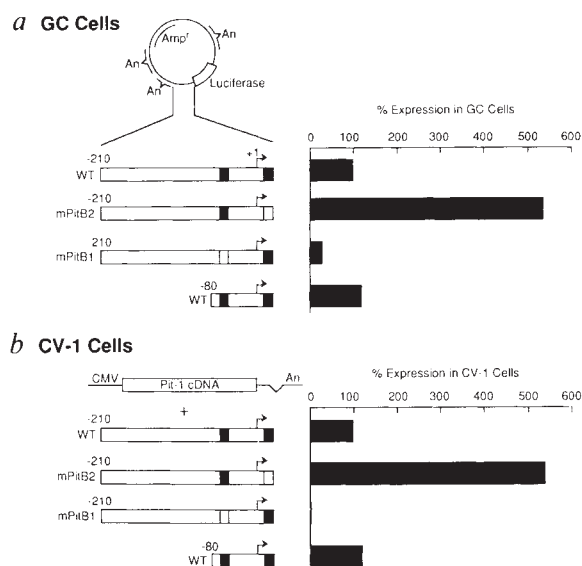
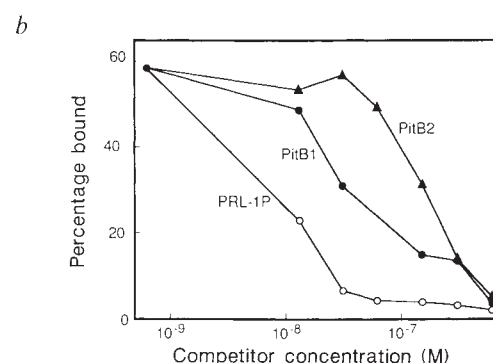
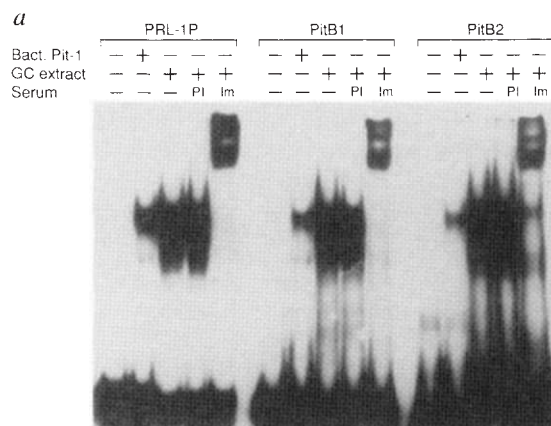


FIG. 3 Binding analysis of the Pit-1-binding element in *pit-1* promoter. **a**, Gel-shift assay of Pit-1 binding to PRL-1P site 5'-CCTGA-TTATA-TATATA-TTCAT-GAAGGT-3' in the prolactin gene promoter and the PitB1 (-70 to -38) and PitB2 (+4 to +34) sites in the *pit-1* promoter using Pit-1-producing bacterial extract or GC nuclear extract. Where indicated, preimmune (PI) or Pit-1 antisera (Im) were added before gel-shift. **b**, Relative affinity of PitB1 and PitB2 binding to the PRL-1P site using bacterially expressed Pit-1 and increasing concentrations of competitor DNA.

METHODS. All binding assays were performed and analysed as previously described¹⁹ using 2–3.5 fmol double-stranded oligonucleotides. Preimmune serum (PI) or anti-Pit-1 serum (Im) were added to the binding reaction for another 10 min. The relative affinity was measured by adding unlabelled competitor oligonucleotide PRL-1P, PitB1 or PitB2 with labelled PRL-1P probe. To quantify competition, labelled protein-DNA complex and unbound probe were measured as previously described¹⁹.

FIG. 4 Analysis of *pit-1* promoter expression in GC and CV-1 cells. **a**, Expression of *pit-1* promoters in GC cells. Reporter plasmids contained the wild-type *pit-1* 5' flanking sequence (-210 to +34) or the *pit-1* 5' flanking sequence with mutated PitB1 (mPitB1) or PitB2 (mPitB2) sites as indicated. Pit-1-binding sites were altered from TTATACAT to TTAAGGCT (PitB1) (antisense) and from ATATACAT to ATATACCT (PitB2) (antisense). Data shown is normalized with that of the reporter plasmid containing the wild-type *pit-1* promoter. **b**, Cotransfection of Pit-1 expression vectors (5 μ g) and *pit-1* luciferase reporter genes (5 μ g) in CV-1 cells. The Pit-1-dependent expression in CV-1 cells was quantified by subtracting the activity of reporter plasmids cotransfected with a control plasmid containing no insert or the *pit-1* cDNA in antisense orientation and normalized with that of the wild-type *pit-1* promoter. **c**, Transcription analysis *in vitro* of *pit-1* promoter. Left, bacterially produced Pit-1 stimulates transcription expression of the mutated *pit-1* promoter in HeLa-cell nuclear extracts. Pit-1 protein was added to transcription assays *in vitro* with the mutant *pit-1* promoter (mPitB2-210) or rat prolactin promoter (Pr1-173). Right, transcription of the wild-type (WT)

Pit-1-210) and mutant (mPitB2-210) promoters in GC and HeLa-cell nuclear extracts. RSV and Pit-1 transcripts are indicated.

METHODS. Reporter plasmids were constructed by inserting different fragments of the *pit-1* promoter with *Bam*HI ends made by the polymerase chain reaction 5' of the firefly luciferase gene²⁷. Expression or control plasmids were constructed by placing *pit-1* cDNA in sense or antisense orientation, in an expression vector under control of the cytomegalovirus (CMV) intermediate-early enhancer promoter and the Simian Virus 40 enhancer. Transfections and luciferase assays were performed as previously described 48 h after transfection^{27,28}. Transcription *in vitro* of wild-type *pit-1* promoter (WT Pit-210) and the *pit-1* promoter with mutated PitB2 site (mPitB2-210) were carried out as previously described¹⁰. The mPitB2-210 promoter and prolactin gene promoter (-173 to +33) were transcribed in 150 μ g HeLa-Cell nuclear extract added with 0, 1, 3 and 5 μ l partially purified bacterially expressed Pit-1. An, polyadenylation signal; Amp^r, ampicillin resistance gene.

contrast, mutation of the PitB2 element did not alter the basal expression of the *pit-1* promoter in HeLa extract (Fig. 4c), suggesting that the PitB2 element depends on a pituitary cell-specific factor, likely to be Pit-1. The addition of bacterially expressed Pit-1 to HeLa-cell nuclear extract increased transcription of a *pit-1* fusion gene containing the mutant PitB2 element in the context of -210 bp of 5' flanking information of the *pit-1* gene (Fig. 4c), at levels comparable to activation of the fusion genes under control of the -173 bp rat prolactin promoter. The transfection and *in vitro* data suggest that occupancy of the 5' (PitB1) site acts to stimulate markedly *pit-1* promoter transcription, whereas occupancy of the PitB2 site in the context of the intact *pit-1* promoter, acts to attenuate the stimulatory effects of the PitB1 site, reflecting either decreased efficiency of transcriptional initiation or attenuation of nascent transcripts.

Evidence for similar autoregulatory mechanisms have been obtained for the distantly related class of developmental factors—the *Drosophila* homeodomain proteins—such as those encoded by *deformed*, *Ultrabithorax* and *fushi tarazu*—demonstrating that these proteins are also capable of binding and activating their own promoters^{20–22}. Other classes of developmental regulators, including zinc-finger DNA-binding proteins (the *hunchback* protein)²³ and the myogenic determination factor MyoD1 (ref. 24), can also autoregulate their own expression. A functional high-affinity binding site downstream of the transcription start site occurs in transcribed regions of other genes²⁰.

Thus, at the high levels of Pit-1 present in mature pituitary cells, the presence of two autoregulatory elements apparently results in Pit-1-activated transcription of the *pit-1* promoter, but with diminished efficacy compared with other Pit-1 target genes. The observation that a genetically dwarf mouse containing a normal *pit-1* transcription unit, but encoding a defective Pit-1 protein, fails to express detectable levels of *pit-1* transcripts (S. Li *et al.* manuscript submitted), is consistent with the proposed autoregulation of the *pit-1* promoter. But the initial activation of the *pit-1* gene cannot be explained by the actions of Pit-1 protein itself or proteins associated with Pit-1. The molecular basis for the initial activation of the *pit-1* gene therefore remains to be elucidated. □

Structure of Arc repressor in solution: evidence for a family of β -sheet DNA-binding proteins

Jan N. Breg, Joost H. J. van Opheusden,
Maurits J. M. Burgering, Rolf Boelens
& Robert Kaptein

Department of Chemistry, University of Utrecht, Padualaan 8, 3584 CH, Utrecht, The Netherlands

THE Arc repressor^{1–3}, which is involved in the switch between lysis and lysogeny of *Salmonella* bacteriophage P22, does not belong to any of the known classes of DNA-binding proteins^{4,5}. Mutagenesis studies⁶ show that the DNA-binding region is located in the 15 N-terminal amino-acid residues. We have now determined the three-dimensional structure of the Arc dimer from an extensive set of interproton-distance data obtained from ¹H NMR spectroscopy. *A priori*, intra- and inter-monomer nuclear Overhauser effects (NOEs) cannot be distinguished for a symmetric dimer. But by using the homology with the *Escherichia coli* Met repressor⁷ we could interpret the NOEs unambiguously in an iterative structure refinement procedure. The final structure satisfies a large set of NOE constraints (1,352 for the dimer). It shows a strongly intertwined dimer, in which residues 8–14 of different monomers form an antiparallel β -sheet. A model for the Arc repressor-operator complex can account for all available biochemical and genetic data. In this model two Arc dimers bind with their β -sheet regions in successive major grooves on one side of the DNA helix, similar to the Met repressor interaction. Thus, Arc and Met repressors are members of the same family of proteins, which contain an antiparallel β -sheet as the DNA-binding motif.

The complete proton resonance assignment for Arc repressor has been reported recently^{8,9}. Furthermore, the secondary structure of the protein has been established from many short and medium range NOEs and consists of a β -sheet region of residues 8–14 and two α -helices in the regions 16–29 and 35–45 (refs 8, 9). Normally, the structural analysis by NMR would then proceed by collecting as many NOEs as possible (including long-range NOEs) and computing the three-dimensional structure based on NOE-derived distance constraints with algorithms such as distance-geometry and restrained molecular dynamics^{10,11}. But for Arc repressor the interpretation of the long-range NOEs is ambiguous because of the dimeric nature of the protein. In a symmetric dimer one may have NOEs between protons, say, A–B and A'–B' within both monomers, but *a priori* this cannot be distinguished from the NOEs A–B' and A'–B between protons in different monomers. For Arc, an interpretation of all NOEs as being intra-monomer (for a loosely coupled dimer) failed as it was not possible to obtain a three-dimensional structure by distance-geometry embedding. We have now resolved the problem of intra- versus inter-monomer NOEs by exploiting the homology with another protein, the *E. coli* Met repressor, for which the crystal structure has recently been reported⁷.

The relevant amino-acid sequence of Met repressor is only weakly homologous with Arc and the related P22 repressor Mnt; there are only seven identical amino-acid residues and five conservative replacements (Fig. 1). But the similarity in secondary structure (a β -sheet followed by two α -helices) and the finding that Met repressor binds to DNA with its β -sheet region⁷ prompted us to explore a possible structural homology with Arc for the purpose of distinguishing intra- and inter-monomer NOEs. We argued that the assumption of structural homology could be validated if this would lead to a structure for Arc that satisfies the extensive set of observed NOEs (676 for the monomer or 1,352 for the dimer). This procedure is reminiscent

Received 2 January; accepted 1 June 1990.

- Ingraham, H. A. *et al.* *Cell* **55**, 519–529 (1988).
- Bodner, M. *et al.* *Cell* **55**, 505–518 (1988).
- Ko, H.-S., Fast, P., McBride, W. & Staudt, L. M. *Cell* **55**, 135–144 (1988).
- Müller, M. M., Rupert, S., Schaffner, W. & Matthias, P. *Nature* **236**, 544–551 (1988).
- Clerc, R. G., Corcoran, L. M., LeBowitz, J. H., Baltimore, D. & Sharp, P. A. *Genes Dev.* **2**, 1570–1581 (1988).
- Scheideit, C. *et al.* *Nature* **336**, 552–557 (1988).
- Sturm, R. A., Das, G. & Herr, W. *Genes Dev.* **2**, 1582–1599 (1988).
- Finney, M., Ruvkun, G. & Horvitz, H. R. *Cell* **55**, 757–769 (1988).
- Herr, W. *et al.* *Genes Dev.* **2**, 1513–1515 (1988).
- Mangalam, H. J. *et al.* *Genes Dev.* **3**, 946–958 (1989).
- Crenshaw III, E. B., Kalla, K., Simmons, D. M., Swanson, L. W. & Rosenfeld, M. G. *Genes Dev.* **3**, 959 (1989).
- Simmons, D. W. *et al.* *Genes Dev.* **4**, 962–979 (1990).
- Cooke, N. E., Brook, F. R. & Frawley, L. L. *Endocrinology* **117**, 187 (1985).
- He, X. *et al.* *Nature* **340**, 35–42 (1989).
- Dollé, P. *Cell* **60**, 809–820 (1990).
- Nelson, C., Albert, V., Elsholtz, H. P., Lu, L. E.-W. & Rosenfeld, M. G. *Science* **236**, 14000–14405 (1988).
- Elsholtz, H. P., Albert, V. R., Treacy, M. N. & Rosenfeld, M. G. *Genes Dev.* **4**, 43–51 (1990).
- Montminy, M. R. & Bilezikjian, L. M. *Nature* **328**, 175–178 (1987).
- Ingraham, H. A. *et al.* *Cell* **61**, 1021–1033 (1990).
- Krasnow, M. A., Saffman, E. E., Kornfeld, K. & Hogness, D. *Cell* **57**, 1031–1043 (1989).
- Biggin, M. D. & Tjian, R. *Cell* **53**, 699–711 (1988).
- Kuziora, M. A. & McGinnis, C. *Cell* **55**, 477–485 (1988).
- Treisman, J. & Desplan, C. *Nature* **341**, 335–337 (1989).
- Thayer, M. J. *et al.* *Cell* **58**, 241–248 (1989).
- Ausubel, F. M. *et al.* *Current Protocols in Molecular Biology*, 641–644 (Wiley-Interscience, New York, 1987).
- Sanger, F., Nicklen, S. & Coulson, A. *Proc. natn. Acad. Sci. U.S.A.* **74**, 5463–5467 (1977).
- de Wet, J. R., Wodd, K. V., DeLuca, M., Helinski, D. R. & Subramani, S. *Molec. cell. Biol.* **7**, 725–737 (1987).
- Chen, C. & Okayama, H. *Molec. Cell. Biol.* **7**, 2745–2752 (1987).

ACKNOWLEDGEMENTS. We are particularly grateful to J. Voss for preparing and providing anti-Pit-1 sera, S. Flynn for assistance, C. Nelson for maintaining the cell culture, M. Kapiloff and M. Mathis for providing bacterially expressed Pit-1 and CREB proteins, Dr J. Boulter for the gift of rat genomic library, and S. Inglis for typing this manuscript. M.N.T. is an EMBO Fellow. This study was supported by the National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases.

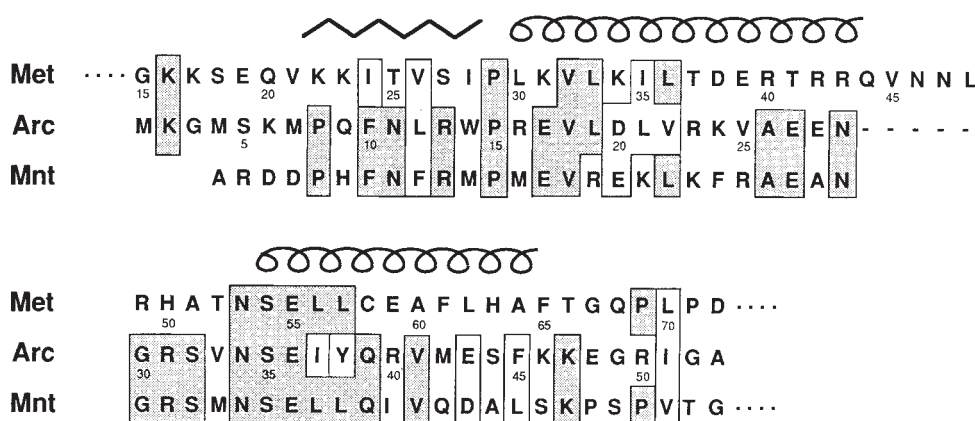


FIG. 1 Amino-acid sequences of the Met, Arc and Mnt repressors (single-letter code). The homology between Arc and Mnt was taken from ref. 4. The alignment with Met repressor is partly based on amino-acid identities and partly on the secondary structure (β -sheet and two α -helices) known for Met from the crystal structure⁷ and for Arc from NMR^{8,9}. For Met and Mnt only the part of the sequence corresponding to that of Arc is shown. Amino-acid identities are in shaded boxes, conservative changes are only boxed.

of that of molecular replacement to solve the phase problem in X-ray crystallography.

The alignment of the sequences of Met with Arc and Mnt (Fig. 1) is based partly on amino-acid residue identity and partly on the estimated start of the α -helices. This led to a deletion of five residues in the Met sequence, shortening the first α -helix by one turn. Using computer graphics model building techniques the amino-acid side chains of Met were replaced by those of Arc, and the loop between helices A and B was reconnected. The structure thus obtained was then used to discriminate between intra- and inter-monomer NOEs. This could be done for some NOEs between helices A and B and those from Trp 14 to helix A, which were all clearly intra-monomer. Several NOEs from helix A to helix B and from helix B to the C terminus were interpreted as inter-monomer. But most of the NOEs from the β -sheet region to helix B could not be interpreted at this stage, as these distances in the model were all too large, irrespectively of the interpretation as intra- or inter-monomer. The structure was then refined in an iterative procedure using restrained molecular dynamics, which allowed the identification of a successively larger number of NOE distance constraints. This led to a complete classification of all 676 NOEs in terms of their intra- or inter-monomer character and a structure that

satisfied virtually all distance constraints. The diagonal plot of Fig. 2 shows extensive inter-monomer contacts within the anti-parallel β -sheet, between the β -sheet and the N-terminal part of helix B and between helices A and B. As a final check the same set of NOE-derived distance constraints was used in distance geometry calculations. A single family of structures was obtained with average r.m.s. differences for backbone atoms (residues 8–45) of 1.3 Å among six structures and a tertiary fold that is identical to that of the Met-derived structure. The resulting structure of the Arc dimer is shown in Fig. 3a. Although the N-terminal and C-terminal residues are disordered, the central core (residues 8–45) is highly symmetric with 0.5 Å r.m.s. difference for backbone atoms between two monomers. A superposition of the Arc backbone atoms of residues 8–28 and 32–45 with those of the Met repressor residues 22–42 and 51–64 according to the alignment of Fig. 1, gave an r.m.s. difference of 2.2 Å. Surprisingly, a much better superposition was obtained (r.m.s. difference 1.2 Å) if residues 52–65 of Met were overlayed with residues 32–45 of Arc. This frame shift of one residue corresponds with a rotation of helix B by 100° (Fig. 3b), and is clearly the result of the NOE constraints, in particular those between β -sheet residues and the B helix.

Finally, it was possible to model the Arc repressor-operator

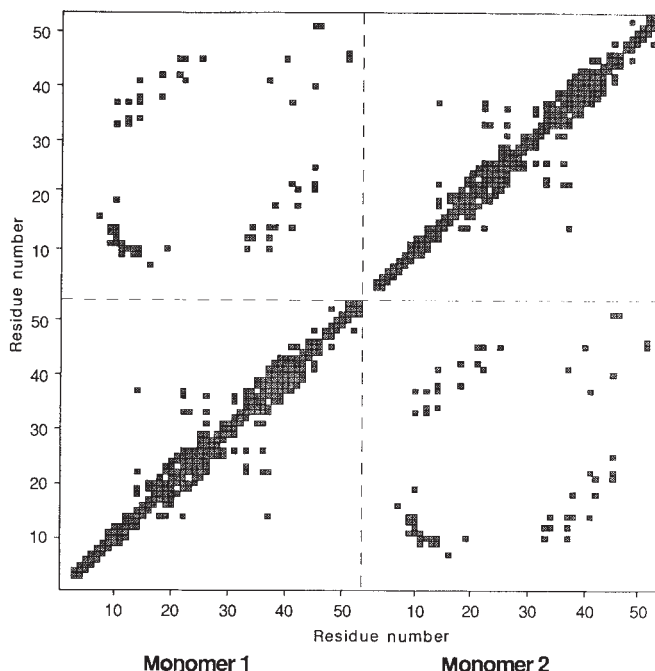


FIG. 2 Diagonal plot showing NOEs observed for the Arc repressor dimer. Each entry represents the presence of one or more NOEs between residues. Most of the long range NOEs are between different monomers (located in the upper left and lower right panel). The lower left and upper right panels are identical and represent intra-monomer NOEs.

FIG. 3 Structure of the Arc repressor dimer. *a*, Stereo diagram of the protein backbone shown in ribbon representation with different colours for the two monomers. The view is perpendicular to the antiparallel β -sheet. Only the residues 8–45 of each monomer are shown. The structure was determined from proton-proton distances obtained from initial NOE build-up rates in a series of 2D NOE spectra of Arc in H_2O and D_2O , with mixing times between 0.05–0.2 s using the $Tyr^{38} C^H-C^H$ distance as a reference. The methyl groups of valines 18, 22, 33 and 41 were stereospecifically assigned. For the prochiral protons, pseudo atoms were used with corresponding corrections of the distance constraints¹⁰. A total of 676 NOEs was identified; of these, 276 were intra-residual, 244 sequential and medium range, and 156 long range. Of the long-range NOEs 93 were interpreted as inter-monomer. In addition to the 1352 NOE constraints for the Arc dimer, 84 H-bond constraints were used in the regions of well-defined secondary structure. The structure was obtained after several cycles of restrained molecular dynamics calculations starting from a Met repressor-derived structure (see text). In subsequent runs the distance constraints force constant increased from 1.2 to 12.0 kcal mol⁻¹ Å⁻². The final energy-minimized structure has a very low constraint energy of 127 kcal mol⁻¹ and a maximum distance violation of 0.8 Å. The calculations were performed on a Silicon Graphics IRIS 4D/120GT workstation with the Insight/Discover software of Biosym Technologies, San Diego. *b*, Superposition of backbone atoms of Arc (residues 8–45) and Met (residues 22–65). The C^β atoms of Ser 35 of Arc and Ser 54 of Met are indicated, to demonstrate the rotation of helix B of Arc compared with that of Met by $\sim 100^\circ$.

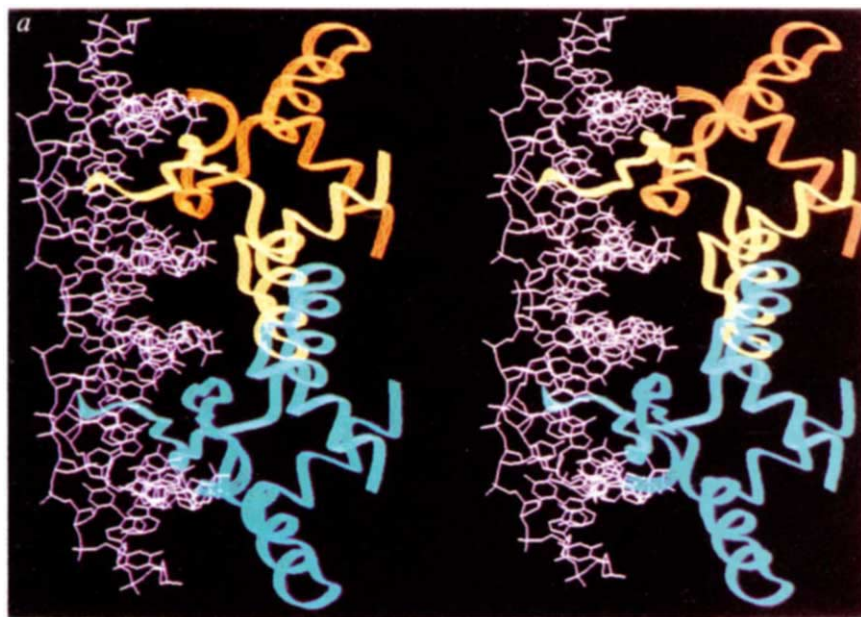
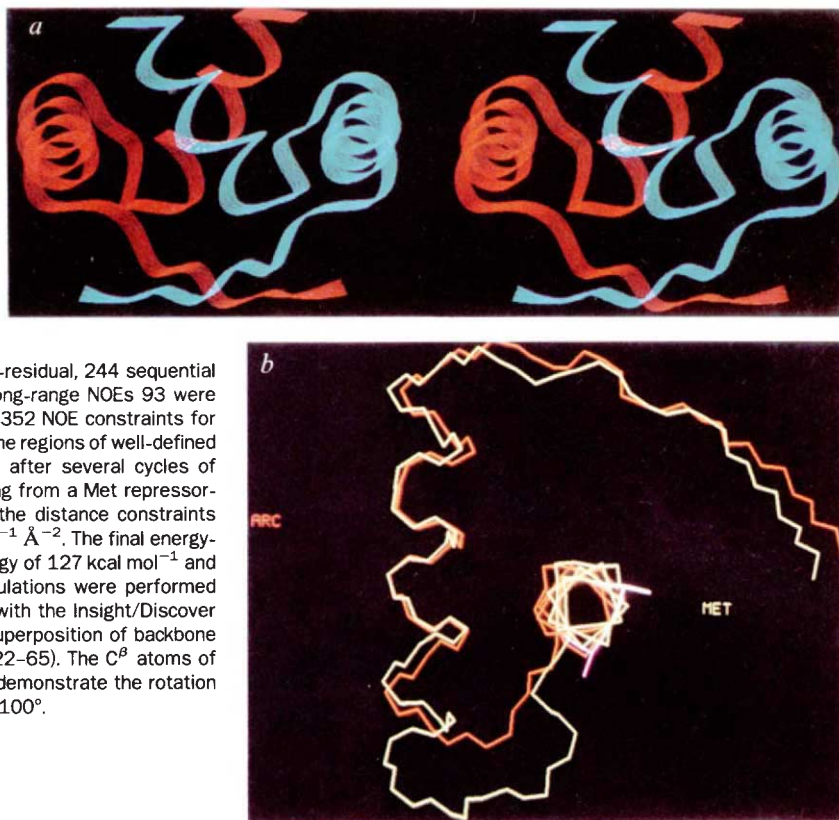
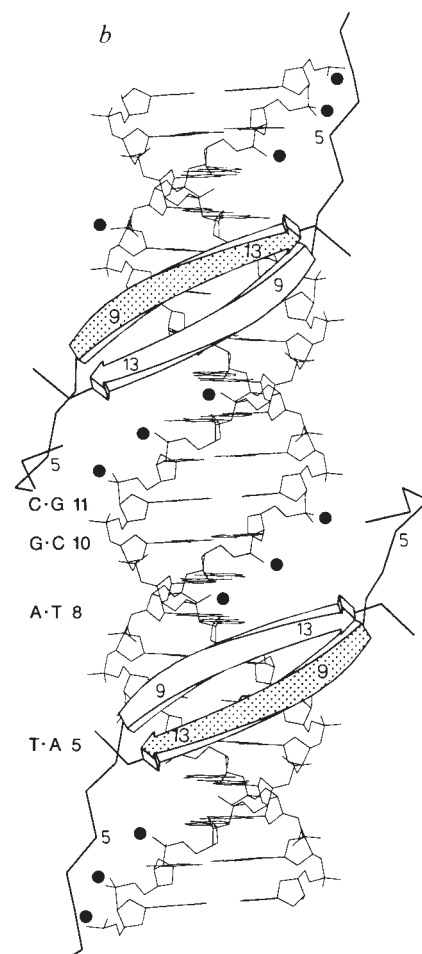


FIG. 4 *a*, Stereo diagram of model of the Arc repressor-operator complex. The view is along the β -sheets of the Arc dimers and the major grooves of the DNA, which is tilted by 30° . The four Arc monomers are shown in ribbon representation, each in a different colour. In addition to the part of the protein shown in Fig. 3*a*, residues 1–7 are included in a conformation consistent with the phosphate ethylation interference data^{16,17}. The model is obtained by docking two Arc dimers on the 21-base pair Arc operator in a regular B-DNA conformation. The antiparallel β -sheets (together with the N-terminal octapeptide identified as the DNA-binding region⁴) clearly protrude from the protein cores and interact with the major groove of DNA. Dimer-dimer contacts are possible, involving residues in the loop between helices A and B. *b*, Expanded view of DNA-binding region of Arc repressor in the complex with Arc operator. The β -sheets of both dimers cover the most important base pairs 5–8 (TAGA) and 14–17 (TCTA). Specific contacts, as found for Mnt repressor, would involve the Arc residues Gln 9 interacting with T·A 5 and Arg 13 with A·T 8. Filled circles indicate phosphates which on ethylation interfere with Arc repressor binding¹⁷. Interactions with residues mainly in the N-terminal peptide 1–8 account for this. Although the complex has rough overall C_2 symmetry, with the dyad axis going through base pair C·G 11, the symmetry of the individual dimers is broken on complex formation.



complex on the basis of the solution structure of the protein (Fig. 3) and data available from mutant studies on Arc and the homologous Mnt repressor¹²⁻¹⁵, from methylation protection¹⁵ and from phosphate ethylation interference experiments^{16,17}. Arc binds to its operator as a tetramer (B. Brown and J. U. Bowie, cited in ref. 4). The model is shown in Fig. 4a, while Fig. 4b gives a more detailed view of the β -sheet/DNA interaction. Two Arc dimers bind in successive major grooves of the operator with the possibility of dimer-dimer contacts involving residues in the loop region between the two α -helices.

Specific protein-DNA contacts are known for Mnt repressor, which must bind to DNA in essentially the same way as Arc does. The best evidence for this comes from experiments in which a hybrid Arc-Mnt protein with the six N-terminal residues of Mnt replaced by the nine N-terminal ones of Arc was shown to have the DNA-binding specificity of Arc¹³. Also, the two-dimensional NOE spectra of Mnt indicate that the secondary structure and tertiary fold of Arc and Mnt are the same (M. J. M. B., unpublished results). Therefore, it is likely that contacts to base pairs 5 and 8 made by the Mnt residues His 6 and Arg 10, respectively⁴, involve the homologous residues Gln 9 and Arg 13 in the case of Arc; these contacts can be formed in the model (Fig. 4b). Furthermore, the N-terminal heptapeptide is sufficiently flexible that it can account for the phosphate ethylation interference data for Arc (compare with Fig. 4b). A further contact found for Mnt repressor¹⁵ between Arg 2 (equivalent to Ser 5 in Arc) and base pairs G-C 10 and C-G 11 is probably not present in Arc (ref. 17). Apparently, the conformation of N-terminal residues is different for Arc and Mnt repressors. We note that in the model shown in Fig. 4b the contacts of Glu 9 and Arg 13 are made by residues of the inner strands with respect to the dyad axis. For Arg 13 this seems to be the only possibility. Alternative models of the complex in which Glu 9 of the other strand contacts the DNA also seem less likely.

Arc, Mnt and Met repressors seem to be members of the same family of β -sheet DNA-binding proteins. In a recent database search for sequence homology with the Arc repressor¹⁸, the Tra Y proteins of the F episomes and related episomes were found as further members of this family. Thus, the antiparallel β -sheet is the latest addition to a repertoire of structural motifs of DNA-binding proteins that includes the helix-turn-helix⁵, zinc-finger¹⁹ and leucine-zipper²⁰ domains. □

Received 2 April; accepted 8 June 1990.

1. Susskind, M. M. *J. molec. Biol.* **138**, 685-713 (1980).
2. Sauer, R. T., Krovatin, W., DeAnda, J., Youderian, P. & Susskind, M. M. *J. molec. Biol.* **168**, 699-713 (1983).
3. Vershon, A. K., Youderian, P., Susskind, M. M. & Sauer, R. T. *J. biol. Chem.* **260**, 12124-12129 (1985).
4. Knight, K. L., Bowie, J. U., Vershon, A. K., Kelley, R. D. & Sauer, R. T. *J. biol. Chem.* **264**, 3639-3642 (1989).
5. Pabo, C. O. & Sauer, R. T. *A. Rev. Biochem.* **53**, 293-321 (1984).
6. Bowie, J. U. & Sauer, R. T. *Proc. natn. Acad. Sci. U.S.A.* **86**, 2152-2156 (1989).
7. Rafferty, J. B., Somers, W. S., Saint-Girons, I. & Phillips, S. E. V. *Nature* **341**, 705-710 (1989).
8. Zagorski, M. G., Bowie, J. U., Vershon, A. K., Sauer, R. T. & Patel, D. J. *Biochemistry* **28**, 9813-9825 (1989).
9. Breg, J. N., Boelens, R., George, A. V. E. & Kaptein, R. *Biochemistry* **28**, 9826-9833 (1989).
10. Wüthrich, K. *NMR of Proteins and Nucleic Acids* (Wiley, New York 1986).
11. Kaptein, R., Boelens, R., Scheek, R. M. & Van Gunsteren, W. F. *Biochemistry* **27**, 5389-5395 (1988).
12. Vershon, A. K., Kelley, R. D. & Sauer, R. T. *J. biol. Chem.* **264**, 3267-3273 (1989).
13. Knight, K. L. & Sauer, R. T. *Proc. natn. Acad. Sci. U.S.A.* **86**, 797-801 (1989).
14. Youderian, P., Vershon, A. K., Bouvier, S., Sauer, R. T. & Susskind, M. M. *Cell* **35**, 777-783 (1983).
15. Knight, K. L. & Sauer, R. T. *J. biol. Chem.* **264**, 13706-13710 (1989).
16. Vershon, A. K., Liao, S.-M., McClure, W. R. & Sauer, R. T. *J. molec. Biol.* **195**, 311-322 (1987).
17. Vershon, A. K., Liao, S.-M., McClure, W. R. & Sauer, R. T. *J. molec. Biol.* **195**, 323-331 (1987).
18. Bowie, J. U. & Sauer, R. T. *J. molec. Biol.* **211**, 5-6 (1990).
19. Evans, R. M. & Hollenberg, S. M. *Cell* **52**, 1-3 (1988).
20. Vinson, C. R., Sigler, P. B. & McKnight, S. L. *Science* **246**, 911-916 (1989).

ACKNOWLEDGEMENTS. We thank R. T. Sauer for helpful discussions and for a sample of Arc repressor used in the initial stage of this work, and S. E. V. Phillips for the coordinates of Met repressor. This work was supported by the Netherlands Foundation for Chemical Research (SON) and the Foundation for Biophysics, with financial aid from the Netherlands Organization for Scientific Research (NWO).

ERRATA

The LDL receptor pathway delivers arachidonic acid for eicosanoid formation in cells stimulated by platelet-derived growth factor

A. J. R. Habenicht, P. Salbach, M. Goerig, W. Zeh, U. Janssen-Timmen, C. Blattner, W. C. King & J. A. Glomset

Nature **345**, 634-636 (1990).

AN error made during the reproduction of Fig. 3c in this letter resulted in the incorrect designation of symbols. These should be: □, PDGF; ○, PDGF+LDL; △, PDGF+AA. □

Cortical microstimulation influences perceptual judgements of motion direction

C. Daniel Salzman, Kenneth H. Britten & William T. Newsome

Nature **346**, 174-177 (1990).

IN this letter Figs 2 and 3 were transposed. Figure 2 is the two-part graph and Fig. 3 the histogram. □

Copies of articles from this publication are now available from the UMI Article Clearinghouse.

For more information about the Clearinghouse, please fill out and mail back the coupon below.

UMI Article Clearinghouse

Yes! I would like to know more about UMI Article Clearinghouse. I am interested in electronic ordering through the following system(s):

- ☐ DIALOG/Dialorder ☐ ITT Dialcom
☐ OnTyme ☐ OCLC ILL Subsystem

☐ Other (please specify) _____

☐ I am interested in sending my order by mail.

☐ Please send me your current catalog and user instructions for the system(s) I checked above.

Name _____

Title _____

Institution/Company _____

Department _____

Address _____

City _____ State _____ Zip _____

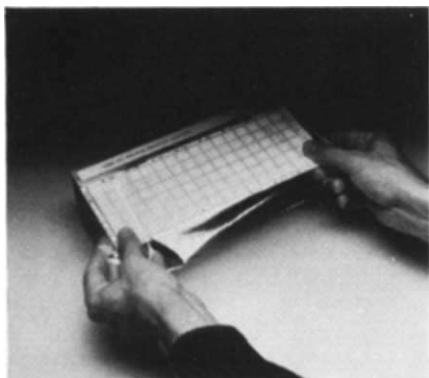
Phone (_____) _____

Mail to: University Microfilms International
300 North Zeeb Road, Box 91 Ann Arbor, MI 48106

Biochemical selection

Budapest, Hungary will be the host city for the 20th meeting of the Federation of European Biochemical Societies, 19–24 August. Featured exhibits include melt-on scintillator for counting beta-labelled samples and an unspecified nuclease that degrades all kinds of DNA/RNA.

MELTILEX melt-on scintillator from Pharmacia is designed for counting beta-labelled samples harvested onto filtermats while eliminating the use of liquid consumables and the problems of liquid waste disposal (*Reader Service No. 100*). It is useful for membrane receptor analysis and other 'on-support' liquid



Pharmacia's 'melt-on' scintillator for 'on-support' counting.

scintillation counting applications where high sample throughput is required. The sheet of MeltiLex is placed on top of the filtermat containing up to 96 samples and then heated for a few minutes at 90 °C on a hot plate. The scintillator melts, is absorbed onto the mat and, on returning to room temperature, solidifies. MeltiLex can be used with conventional liquid scintillation counters or with Pharmacia's 1205 Betaplate flatbed LSC system. The 1205 is designed to cut down on handling times by counting whole dried filtermats and eliminating the need to punch out filter disks.

Cryocool is a **non-volatile heat transfer fluid** for use in stainless steel refrigerated cold traps (*Reader Service No. 101*). It is a



Cryocool — Savant's ethanol/methanol substitute for cold traps.

useful ethanol/methanol substitute because, unlike ethanol and methanol, Cryocool does not need to be replaced or replenished periodically. It exhibits a very low level of water adsorption, so that water and Cryocool are immiscible. When using Cryocool, any water condensed in the trap over time falls to the bottom and freezes. The ice can then be scooped out, leaving clean, non-volatile Cryocool remaining. Cryocool is a non-toxic, odourless fluid that has no special storage requirements. One quart costs \$45 (US).

Peptide profile

Cambridge Research Biochemicals has launched a Cleavable Peptide Kit for **multiple peptide synthesis** without the need for dedicated instrumentation (*Reader Service No. 102*). The kit, which uses a novel polyethylene pin support that was developed at the Commonwealth Serum Laboratories in Australia, allows up to 192 different peptides to be synthesized. Peptides are cleaved in aqueous solution and at physiological pH, and are presented in a form that is suitable for T-cell epitope scanning, peptide analogue studies, solution-phase assays and for the preparation of peptide conjugates — without the need for further purification.

C-C Biotech is introducing a series of novel **glucosaminylmuramyl peptides** (*Reader Service No. 103*). They are synthetic analogues of bacterial cell-wall glycopeptides, which act as modulators of humoral and cellular immunity reactions. According to C-C Biotech, the analogues possess immunoadjuvant and protective activity against bacterial, viral, including tumourigenic infections, and display antimetastatic action and somnogenic effects. *N*-Acetyl-D-glucosaminyl-(B1-4)-*N*-acetylmuramyl-L-alanyl-D-isoglutamine (GMDP) is the first product off the production line.

Pyroglutamate aminopeptidase and restriction protease factor Xa are the latest additions to Boehringer's line of **sequencing-grade proteases** (*Reader Service No. 104*). Sequencing-grade pyroglutamate aminopeptidase is isolated from calf liver and cleaves pyroglutamate from the N-terminus of peptides and proteins; this allows for sequencing of the chain via Edman degradation. Sequencing-grade factor Xa is isolated from bovine



Two sequencing-grade proteases are new from Boehringer.

plasma and cleaves the C-terminus of arginine in rare tetrapeptide sequences. Factor Xa is the first product in a new line of 'restriction proteases' that Boehringer says, are particularly useful for the cleavage of fusion (recombinant) proteins. Both products are tested for purity and cleavage specificity.

DNA/RNA details

New prestained and unstained **molecular weight markers** for electrophoresis are available from Integrated Separation Systems (*Reader Service No. 105*). The markers incorporate an expanded range of proteins from 3,500 to 340,000 Da.



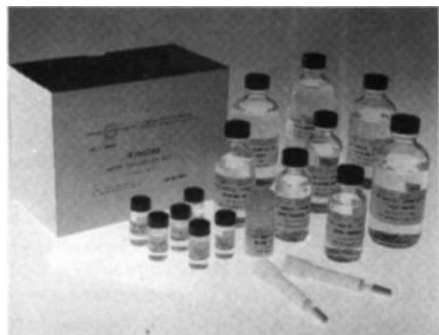
Molecular weight markers come in unstained and prestained forms from ISS.

Low-, mid- and high-range kits are available, both unstained and prestained with red-labelled markers that are readily visible and colour stable. Markers are supplied suspended in sample buffer and ready for loading onto a gel.

Benzonase is an **unspecified nuclease** from E. Merck that degrades all kinds of DNA and RNA; including single- or double-stranded, linear, circular or supercoiled, yielding short (3–5-bp range) oligonucleotides (*Reader Service No. 106*). Benzonase is available in two grades: 99 and 90 per cent pure, costing DM218 and DM148 (FRG), respectively for 10,000 units. The molecular activity of 99 and 90 per cent benzonase is 1×10^6 units per mg protein and 4×10^5 units per mg protein. The optimum pH operating

range of benzonase is 7.8–9.2 and the optimum temperature is 37 °C.

RiboSep from Collaborative Research is a kit designed for the **isolation of high-quality mRNA** directly from cell or tissue lysates (*Reader Service No. 107*). The ready-to-use kit includes oligo (dT) cellulose, columns, buffers, DEPC-treated water, NaCl, NaOAc, proteinase K and instructions for obtaining poly (A)⁺-rich RNA. Sufficient reagents are included for up to four purifications. Typically, yields of 40–50 µg of mRNA from 10⁸ cells or



RiboSep — CRI's complete kit for mRNA isolation in 4 hours.

one gram of tissue are possible, says the company. The \$210 (US) RiboSep kit is designed to produce high-quality intact mRNA in four hours.

Advanced Magnetics has introduced a line of BioMag products for the **magnetic isolation of DNA and RNA** (*Reader Service No. 108*). Oligo (dT), streptavidin, biotin and avidin have been conjugated to the 1-µm BioMag Superparamagnetic Particle to allow the quick and safe separation of labelled DNA or RNA. Biomag products can be used in DNA probe assays, as an alternative to nitrocellulose in hybridization reactions, and to purify DNA binding



Advanced Magnetics provides a magnetic means of isolating DNA/RNA.

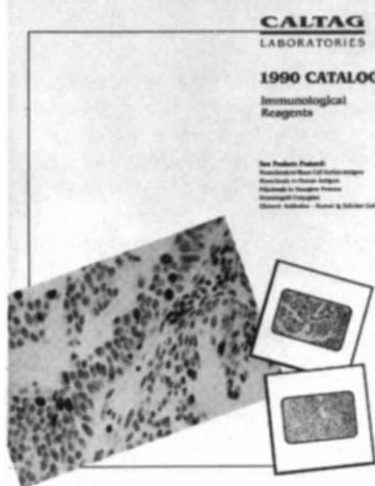
proteins. Advanced Magnetics' products can be used in conjunction with the company's 16-tube capacity magnetic separation unit, which holds both 1- and 1.5-ml tubes and which ensures the isolation of the conjugated magnetic particle.

Stratagene has a new **triple helix cosmid vector** that is designed to reduce the time and effort spent mapping cosmid clones (*Reader Service No. 109*). The procedure involves the complete digestion with *Not* I, followed by partial digestion with

the enzyme of interest. These fragments are then complexed with one or two triplex-forming labelled oligonucleotide probes and electrophoresed into an agarose gel. Autoradiography from the dried gel eliminates the need to transfer and probe the fragments on a solid support, which results in significant time savings, says Stratagene. The \$240 (US) triple-helix vector is suitable for cloning limited amounts of DNA and may be used for rapid genomic walking and mammalian gene transfer.

Product news

The 1990 **catalogue of immunological reagents** from Caltag has 550 product entries, which include affinity-isolated polyclonal antibodies, monoclonal antibodies and related products (*Reader Service No. 110*). Featured for the first time are monoclonals to mouse cell surface markers, monoclonals to human antigens,



Caltag's catalogue covers 550 immunological reagents.

polyclonals to oncogene proteins, immunogold conjugates and chimeric antibodies.

The free **1990 molecular biology catalogue** from Sigma provides a complete listing of nucleic acid-modifying enzymes, nucleic acids, reagents, equipment and books (*Reader Service No. 111*). New products in the molecular marketplace include pYAC vectors, a DNase- and RNase-free alkaline phosphatase preparation, deoxy- and dideoxy-NTPs for sequencing, microbiological media and antibiotics, which are supplied in pre-weighed vials. For user-reference, the catalogue contains restriction sequence tables and plasmid restriction maps.

The **Serva catalogue for molecular biology** is the first in a series of publications that will focus on a specific product area (*Reader Service No. 112*). Featured in the catalogue with accompanying technical information, tables and figures are DNA/RNA modifying enzymes, enzymes and

inhibitors for the isolation of DNA/RNA, 83 restriction enzymes, and nucleic acids and nucleotides. Kits for cDNA synthesis, cloning, expression, sequencing and packaging into lambda phages are available — all supplied with detailed instructions. Several sequencing primers, one cDNA synthesis primer, and two new lytic enzymes — achromopeptidase and kitase — complete the line up.

Reagents for immunology

Zymocel from Alpha-Beta Technology is a highly purified preparation (> 95 per cent pure) of β-glucan, which is known to

ADVERTISEMENTS

Custom DNA

Purified and Shipped in 48 hours,
Worldwide.

100 ug. \$10.00 a base for the first 15 bases,
\$7.50 for each additional base.
Delivered.

Research Genetics

1-800-533-4363

In the United Kingdom 0-800-89-1393

In Canada 1-800-533-4363

FAX 205-536-9016

Reader Service No.26

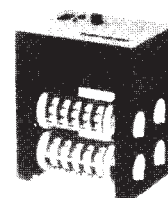
ANTICOAGULANTS and CONNECTIVE TISSUE PRODUCTS

BiopharmTM
Biochemicals

The 1990/91 catalogue contains a variety of high quality
enzymes and protease inhibitors unique to Biopharm
SEND FOR YOUR FREE CATALOGUE NOW

Reader Service No.18

DIABORM EQUILIBRIUM DIALYSIS SYSTEM



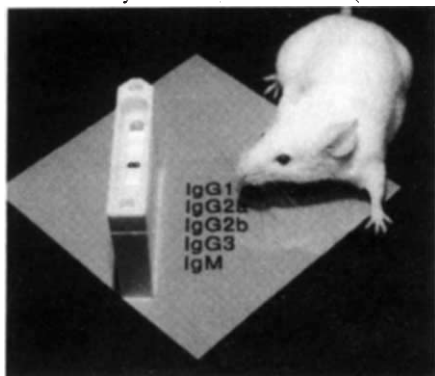
The gentlest method to quantify
the binding parameters and
thermodynamic data between
ligands and biopolymers.
Results come closest to
the actual values.
Sample volume from
0.2 ml to 5.0 ml.
Up to 20 experiments
in parallel.

DIABORM
Geräte
P.O. Box 650126
D-6000 München 55,
FRG.

Reader Service No.49

stimulate macrophages and neutrophils (*Reader Service No. 113*). According to the company, Zymocel induces responses — phagocytosis, the respiratory burst, leukotriene and lysosomal enzyme production, and the synthesis of cytokines (IL-2, TNF, GM-CSF) — in monocytes and neutrophils mediated by the β -glucan receptor. Zymocel is supplied as a dry powder in 20- and 100-mg quantities for \$45 and \$120 (US), respectively. It is also available as a sterile pyrogen-free suspension: 100 mg costs \$135 (US).

Iso-stat, the membrane-based enzyme immunoassay **isotyping kit** from Immunodiagnostic Systems, is designed for the rapid determination of mouse monoclonal antibody class and sub-class (*Reader*



Iso-stat mouse monoclonal isotyping kit.

Service No. 114). The EIA uses affinity-purified, isotope-specific antibodies covalently bound to a test membrane. IgG1, IgG2a, IgG2b, IgG3 and IgM can be differentiated on a single test cartridge. The isotype of the immunoglobulin is determined by the position of the colour development in the cartridge. Results can be achieved in only five minutes, says IDS. Each Iso-stat kit contains sufficient reagents for 36 isotype determinations.

Nycomed's now sells its **lymphocyte separation medium** — Lymphoprep — pyrogen-free (*Reader Service No. 115*). Lymphoprep can be used for the *in vitro* isolation of human bone marrow cells, preparation of lymphocyte suspensions for tissue typing, and production of anti-lymphocyte sera. It is formulated as a sodium metrizoate/Ficoll mixture to allow one-stage lymphocyte separation. Centrifugation at 800 g for 15 minutes at 20 °C produces cell yields of 95 per cent, says Nycomed.

Six new **anti-mouse monoclonals** for one-, two-, three- and four-colour analysis are available from Coulter (*Reader Service No. 116*). They include Thy 1.2 (Pan T), Ly 2 (CD8a), L3/L4 (CD4), Ly 1.2 (T cells), Macph (Macrophages) and Ly 5 (B cells). These anti-mouse mAbs are optimized for flow cytometry and are



Anti-mouse monoclonals from Coulter. free of unconjugated proteins, aggregates and free fluorochromes. These preparations are available in various product forms, which include FITC (fluorescein isothiocyanate) conjugates, RD1 (phycoerythrin) conjugates and biotin.

Liposorb is a new reagent from Calbiochem for the **removal of lipoproteins from serum and plasma** (*Reader Service No. 117*). Liposorb selectively removes lipoproteins without the activation of coagulation factors. It is chemically stable in acid/alkaline, can be decomposed enzymatically, is thermally stable and can be sterilized. □

These notes are compiled by Diane Gershon from information provided by the manufacturers. To obtain further details, use the reader service card bound inside the journal. Prices quoted are sometimes nominal, and apply only within the country indicated.

From the publishers of
Nature and Bio/Technology

GENE TRANSFER AND EXPRESSION

A Laboratory Manual

By **Michael Kriegler, Ph.D.**, Senior Scientist, Department of Molecular Biology, Cetus Corporation, California

...

Send orders to Globe Book Service Ltd., FREEPOST, Stockton House, 1 Melbourne Place, London WC2B 4 LF, UK. Or call: 01-379-4687. We accept Access/MasterCard, Visa, Diners' Club and American Express. Please add £2.00 postage and packing. FREEPOST applies to UK only. (Globe Book Services is a division of Macmillan, Publishers, Ltd.)

Gene Transfer And Expression: A Laboratory

Manual is an up-to-date manual that describes recent techniques in performing gene transfer and measuring gene expression. The introduction includes a descriptive catalogue of numerous viral and cellular cis-acting elements as well as state of the art mammalian expression vectors. It discusses the process of transferring genes into mammalian cells and subsequently assaying for the transfer and expression of the genes of interest.

Featuring

Expression Cloning and SIB Selection Techniques • Subtractive Hybridization Techniques • Retrovirus Mediated Gene Transfer

Also including

The Propagation of Cells and Cell Lines • DNA Transfer Techniques • Drug Selection and Gene Amplification Techniques • Expression Cloning • Subtractive Hybridization • Retrovirus Mediated Gene Transfer Techniques • Highly Sensitive Assays For Gene Transfer and Expression • Detection of DNA and RNA by Both Conventional and PCR Based Techniques • Western Techniques Employing the Latest Isotopic and Non-Isotopic, Detection Methods

October 1990, 240 pp., 0-333-53543-X, £27.95

M-